Am J Physiol Heart Circ Physiol 283: H2371–H2378, 2002.
First published August 22, 2002; 10.1152/ajpheart.00383.2002.

Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis

XUE ZHAO, XIANGRU LU, AND QINGPING FENG
Cardiology Research Laboratory, Lawson Health Research Institute, London Health Sciences Centre, Departments of Medicine, Physiology, and Pharmacology, University of Western Ontario, London, Ontario, Canada N6A 4G5

Received 1 May 2002; accepted in final form 13 August 2002

Zhao, Xue, Xiangru Lu, and Qingping Feng. Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis. *Am J Physiol Heart Circ Physiol* 283: H2371–H2378, 2002. First published August 22, 2002; 10.1152/ajpheart.00383.2002.—We recently demonstrated that mice deficient in endothelial nitric oxide (NO) synthase (eNOS) have congenital septal defects and postnatal heart failure. However, the mechanisms by which eNOS affects heart development are not clear. We hypothesized that deficiency in eNOS impairs myocardial angiogenesis. Myocardial capillary densities were measured morphometrically in neonatal mouse hearts. In vitro tube formation on Matrigel was investigated in cardiac endothelial cells. In vivo myocardial angiogenesis was performed by implanting Matrigel in the left ventricular myocardium. Myocardial capillary densities and VEGF mRNA expression were decreased in neonatal eNOS$^{-/-}$ compared with neonatal wild-type mice ($P < 0.01$). Furthermore, in vitro tube formation from cardiac endothelial cells and in vivo myocardial angiogenesis were attenuated in eNOS$^{-/-}$ compared with wild-type mice ($P < 0.01$). In vitro tube formation was inhibited by N^G-nitro-L-arginine methyl ester in wild-type mice and restored by a NO donor, diethylenetriamine-NO, in eNOS$^{-/-}$ mice ($P < 0.05$). In conclusion, deficiency in eNOS decreases VEGF expression and impairs myocardial angiogenesis and capillary development. Decreased myocardial angiogenesis may contribute to cardiac abnormalities during heart development in eNOS$^{-/-}$ mice.

heart; endothelial cells; vascular endothelial growth factor; knockout mice; capillary development

ANGIOGENESIS is the development of new blood vessels from preexisting blood vessels, a complex process involving dissolution of basement membrane underlying endothelial cells, endothelial cell migration, adhesion, proliferation, and organization into tubes, followed by lumen formation (26, 28). Vascularization occurs concomitantly to organ growth, and angiogenesis is an event involved closely in organ development and tissue repair. Organized blood vessel formation is essential for physiological function of organs. Angiogenesis is a multistep process controlled by the balance of pro- and antiangiogenic factors (26, 28).

Nitric oxide (NO) production from endothelial NO synthase (eNOS) plays an important role in normal fetal development. Mice deficient in eNOS show fetal growth restriction, reduced survival, and an increased rate of limb abnormalities (14). Furthermore, eNOS$^{-/-}$ mice have a high incidence of bicuspid aortic valve (18). We recently demonstrated that a deficiency in eNOS led to increased cardiomyocyte apoptosis, congenital septal defects, and postnatal heart failure (9), suggesting that eNOS is important in fetal heart development. However, the mechanisms by which eNOS affects fetal heart development are not clear. Recent studies have demonstrated that NO production is essential for angiogenesis in hindlimb ischemia (20), wound healing (17), and coronary collateral growth after myocardial ischemia (19). VEGF is a key mediator of angiogenesis under physiological and pathological conditions (10). Inhibition of VEGF leads to impaired organ development and increased mortality (12). The angiogenic effect of VEGF is predominantly mediated by eNOS (11). NO production from eNOS is not only a downstream mediator of VEGF-induced angiogenesis (20) but also an upstream promoter of VEGF expression (15). It seems that there is a positive feedback mechanism between NO and VEGF that promotes angiogenesis. However, the role of eNOS in myocardial angiogenesis during heart development is still not fully understood. In the present study, we hypothesized that a deficiency in eNOS results in decreases in myocardial VEGF expression and angiogenesis in neonatal hearts. Impaired angiogenesis in the myocardium may contribute to myocardial apoptosis, heart failure, and high mortality in neonatal eNOS$^{-/-}$ mice (9).

MATERIALS AND METHODS

Animals. The animals used in this study were handled in accordance with the guidelines of the Animal Care Committee at the University of Western Ontario, Ontario, Canada. Breeding pairs of eNOS$^{-/-}$ and C57BL/6 wild-type mice were purchased from Jackson Laboratory (Bar Harbor, ME). A breeding program was carried out to produce neonates. Mice

Address for reprint requests and other correspondence: Q. Feng, Dept. of Medicine, London Health Sciences Centre, Victoria Campus, 375 South St., London, Ontario, Canada N6A 4G5 (E-mail: qfeng@uwo.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

were genotyped by a PCR method using genomic DNA extracted from the tail.

Analysis of myocardial capillary densities. Neonatal wide-type C57BL/6 and eNOS$^{-/-}$ mice at postnatal *day 1* were used for stereology analysis of myocardial capillary vasculature similar to previously described methods (29) with modifications. Under a stereomicroscope, the chest was opened from the sternum. An equal volume mixture of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and cardioplegia solution (120 mM NaCl and 30 mM KCl) was slowly infused into the left ventricular chamber using a microsyringe to perfuse the heart. Hearts were removed, placed in cardioplegia for 1 min, fixed with 3% glutaraldehyde in 0.1 M phosphate buffer for another 2 h, and then postfixed in 1% osmium teroxide in phosphate buffer (pH 7.2). The heart samples were embedded with Epon-araldite plastic mixture. A dissecting microscope was used to make sure that the heart was embedded in proper position so that transverse sections were obtained. One-micrometer sections were cut, starting from the apex of the heart along the long axis of the left ventricle. Sections that showed double ventricular chambers were placed on glass slides and stained with Richardson's solution (mixture of 1% azure II in distilled water and 1% methylene blue in 1% sodium borate). Subepicardial regions of the left ventricular free wall on the sections were photographed in sequence by using a digital camera under a microscope (Leitz) at a magnification of ×400. Twelve to fourteen images were taken for one heart section. Capillaries were assessed by the SigmaScan Pro program. Capillaries were defined as those structures possessing a patent lumen formed by a single endothelial cell and usually containing red blood cells. Photographed fields from each heart included a total of ~150–300 capillary profiles. The combined photographed sample fields from each neonatal mouse heart averaged 0.069 mm^2. The number of capillaries in the sample fields was counted and expressed as capillary density per millimeter squared. Measurement of capillary densities in adult mouse hearts was performed in the same way as that in neonatal mice.

Immunohistochemistry. Hearts isolated from wild-type and eNOS$^{-/-}$ neonatal mice were fixed in 10% neutral buffer formaldehyde, embedded in paraffin, and cut into 5-μm sections. Identification of endothelial cells was performed using an antibody against von Willebrand factor (vWF; DAKO). In brief, tissue sections were incubated in 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. To prevent nonspecific binding, sections were preincubated for 30 min in PBS containing horse serum. The sections were then incubated with rabbit anti-human vWF antibody (1:200). The sections were subsequently incubated with swine anti-rabbit IgG antibody (1:100, DAKO), followed by incubation with rabbit proxidase anti-peroxidase complex (1:50, DAKO). Staining for vWF was visualized with 3-diaminobenzidine substrate, which produces a yellow-brown color. Sections were counterstained with hematoxylin.

Isolation and purification of cardiac endothelial cells. Cardiac endothelial cells were isolated using a modification of previously described methods (27). Briefly, after the heparinized (5,000 U/kg) mice were euthanized by cervical dislocation, ventricles were aseptically removed and transferred to ice-cold HBSS. The tissue was minced and incubated with HBSS containing 500 U/ml collagenase II (Worthington), 0.6 U/ml dispase II (Boehringer Mannheim), and 0.1% (wt/vol) BSA (Sigma) for 40 min at 37°C. The digested material was filtered through 100-μm nylon mesh and washed twice. Subsequently, the cells were incubated with microbeads (Dynal beads M-450, Dynal) coated with lectin (Griffonia simplicifolia-1, Sigma) in medium 199 (M199) supplemented with 1% FCS at room temperature for 15 min. Microbeads attached to endothelial cells were captured by Dynal magnet and seeded onto gelatin-coated 35 × 10-mm tissue culture dishes in M199 supplemented with 20% FCS, 50 μg/ml endothelial cell growth supplement (ECGS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml of heparin. Cardiac endothelial cells were grown to confluence before they were passed to Matrigel (BD Matrigel Matrix, BD Biosciences)-coated 96-well plates.

In vitro two-dimensional cardiac endothelial cells culture. Matrigel contains various growth factors including endothelial growth factors, platelet-derived growth factor, insulin growth factor 1, and transforming growth factor-β. Matrigel stored at −20°C was thawed at 4°C overnight. A cooled pipette was used to mix the Matrigel to homogeneity, which was then diluted 1:1 in ice-cold serum-free DMEM. The 96-well plates were coated with the diluted Matrigel (50 μl/well), incubated at 37°C for 1 h, and then washed with serum-free DMEM. Endothelial cells (3×10^4 cells) were seeded onto each well and cultured at 37°C for 4 h in DMEM supplemented with 20% FCS, 25 μg/ml ECGS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml heparin. The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 500 μM, Sigma) or the NO donor diethylenetriamine-NO (DETA-NO; 2 μM, Sigma) was added to the medium at the time of seeding. Cells were observed with an inverted microscope and photographed using a digital camera at ×400 magnification at 4 h after seeding. In vitro angiogenesis was assessed by the formation of capillary-like structures from cardiac endothelial cells on Matrigel, as previously described (4). To measure the formation of the capillary-like network, the number of connections between three or more capillary-like structures was counted and expressed as the number of capillary connections per field. Furthermore, the average thickness of the tube or cell overcrowding, the total length of tubes per field, and the average length (distance) of tube between connections were quantified by image analysis with an image analysis system (SigmaPro).

In vivo Matrigel angiogenesis. In vivo angiogenesis was assessed as the growth of blood vessels from myocardial tissue into a Matrigel plug implanted in wild-type and eNOS$^{-/-}$ mice (10–13 wk). Matrigel was stored at −20°C and thawed at 4°C for 2 h before use (24). The mouse was anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg), intubated, and artificially ventilated with room air. Tidal volume was set at 0.6 ml with 90 breaths/min and a 40/60 inspiration-to-expiration ratio (SAR-830, CWE). Body temperature was maintained at 37°C. A left thoracotomy was performed, and the heart was exposed. Matrigel was injected into the anterior wall of the left ventricle near the apex at a total volume of 8 μl using a 29-gauge needle adapted to a Hamilton microsyringe. To prevent leakage of Matrigel from the hole of puncture, the needle was kept in myocardium for ~30 s after injection. The chest was closed by sutures in layers. Three days after injection, the mice were killed, and the hearts were harvested, fixed in 10% neutral buffered formalin, and embedded in paraffin. Transversal sections (5 μm) were cut sequentially from the apex to base of the heart, stained with hematoxylin and eosin, and examined under a microscope. The vessel area and total Matrigel area were planimetrically assessed from three different sections. Results are expressed as the percentage of the vessel area to the total Matrigel area.

RT-PCR. The mRNA expression of VEGF was determined by RT-PCR similar to the method that we described previously (8). Total RNA was isolated from the left ventricular myocardium with TRIZol reagent (GIBCO-BRL). The RNA was extracted with the use of phenol-chloroform, precipitated by isopropanol, and quantified by spectrophotometry. Subsequently, RNA was reverse transcribed into first-strand cDNA using a Moloney murine leukemia virus reverse transcriptase system. The cDNA was amplified by PCR using a programmable thermal cycler (Progene, Techne; Cambridge, UK). The forward and reverse primers for the mouse VEGF gene (Genbank Accession No. NM009505) were 5′-ACC TCA CCA AAG CCA GCA CA-3′ and 5′-GGC ATG GTG GTG ACA TGG TT-3′, respectively. To ensure that equal amounts of reverse-transcribed cDNA were added to the PCR mixture, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Genbank Accession No. M17701) was also amplified using the following primers: forward primer, 5′-AAA GGG CAT CCT GGG CTA CA-3′; reverse primer, 5′-CAG TGT TGG GGG CTG AGT TG-3′. The logarithmic ranges of amplification were established for VEGF and GAPDH (35 and 25 cycles, respectively) to ensure that the amplified PCR product reflected the original mRNA level. The PCR product was separated on a 2% agarose gel and visualized under UV light. The predicted lengths of the amplification product for VEGF and GAPDH were 334 and 297 bp, respectively. VEGF mRNA expression in relation to GAPDH mRNA was analyzed by densitometry.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed by one-way ANOVA followed by Student-Newman-Keul's multiple-comparison test or unpaired Student's *t*-test where appropriate. $P < 0.05$ was considered to be statistically significant.

RESULTS

Impaired myocardial capillary development in neonatal eNOS$^{-/-}$ mice. To determine the importance of eNOS in myocardial angiogenesis, we first examined myocardial capillary vasculature in cross sections of the left ventricular myocardium in neonatal mice. More abundant capillary lumens were observed in the subepicardial region compared with the middle layer or subendocardial region in both wild-type and eNOS$^{-/-}$ mice. However, there were more well-developed capillaries in wild-type mice (Fig. 1*A*) compared with eNOS$^{-/-}$ mice (Fig. 1*B*). Immunostaining for vWF also revealed an appreciable decrease in subepicardial regions of the left ventricular wall in neonatal eNOS$^{-/-}$ mice (Fig. 1*D*) compared with wild-type mice (Fig. 1*C*). Capillary densities in the subepicardial regions of the left ventricular free wall were decreased by 40.7% in eNOS$^{-/-}$ neonates compared with neonatal wild-type mice ($P < 0.01$, $n = 7$ neonates/group; Fig. 2). However, in the adult left ventricular myocardium, there was no significant difference in capillary density (1,889 ± 157 vs. 2,027 ± 236 capillaries/mm^2) between wild-type

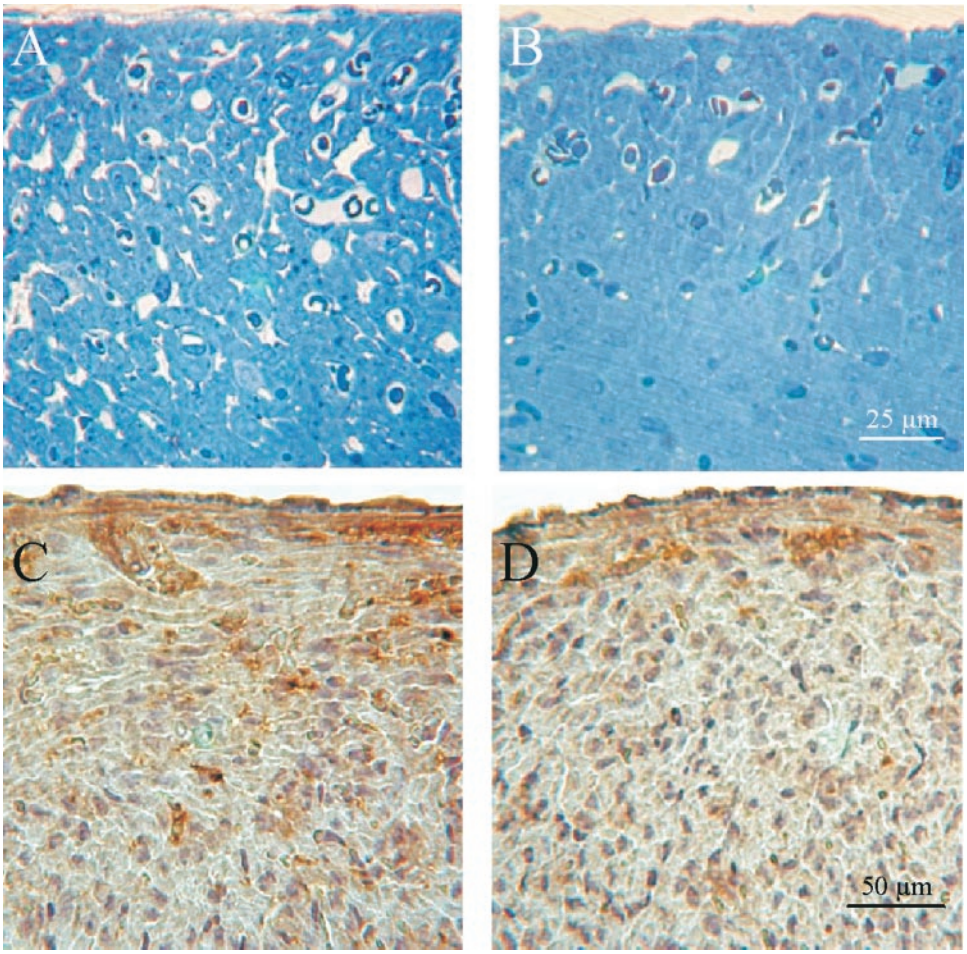

Fig. 1. Micrographs of heart sections stained with Richardson's solution (*A* and *B*) and immunohistochemical staining for von Willebrand factor (*C* and *D*) in neonatal mouse hearts at postnatal *day 1*. *A* and *C*: abundant capillaries in the left ventricular (LV) myocardium of wild-type mice. *B* and *D*: a few and scattered capillaries in the LV myocardium of endothelial nitric oxide (NO) synthase (eNOS)-deficient (eNOS$^{-/-}$) mice.

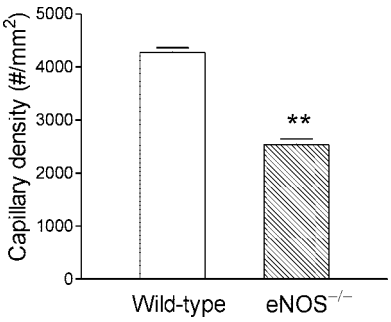

Fig. 2. Capillary densities in the LV myocardium of neonatal mice at postnatal *day 1* (n = 7 mice/group). $^{**}P < 0.01$, eNOS$^{-/-}$ vs. wild-type mice.

and eNOS$^{-/-}$ mice [n = 4 mice/group, P = not significant (NS)].

Impaired in vitro angiogenesis in eNOS$^{-/-}$ cardiac endothelial cells. Having documented the impairment of myocardial capillary development in neonatal eNOS$^{-/-}$ mice, we conducted in vitro angiogenesis of cardiac endothelial cells on Matrigel. Cardiac endothelial cells isolated from five adult mice hearts were pooled onto one 6-well dish as a primary culture. Five independent primary cultures were performed in both wild-type and eNOS$^{-/-}$ mice. The cardiac endothelial cells in the primary culture were allowed to grow to complete confluence before they were seeded onto Matrigel-coated 96-well plates. Tube formation reached the optimal level after 4 h of culture. The effects of NO on angiogenesis in cardiac endothelial cells are shown by the representative images in Fig. 3. Compared with the wild type (Fig. 3*A*), tube formation was markedly decreased in eNOS$^{-/-}$ cardiac endothelial cells (Fig. 3*B*). Treatment with L-NAME decreased tube formation in wild-type cardiac endothelial cells (Fig. 3*C*), whereas DETA-NO increased tube formation in eNOS$^{-/-}$ cardiac endothelial cells (Fig. 3*D*). Quantitative analysis showed that the total length of tube between connections, the average thickness of tube, and the number of capillary connections were significantly decreased in eNOS$^{-/-}$ compared with wild-type cardiac endothelial cells (Fig. 4, *A–C*; $P < 0.05$, n = 5 cells/group). Treatment with L-NAME in wild-type cardiac endothelial cells for 4 h caused a significant inhibition in total tube length, average tube thickness, and number of connections by 27.9%, 25.1%, and 31.6%, respectively (Fig. 4; $P < 0.05$). When DETA-NO was added to the medium for 4 h, the ability of cardiac endothelial cells to form capillary-like structures was significantly restored in eNOS$^{-/-}$ mice (Fig. 4; $P < 0.05$).

Impaired in vivo myocardial angiogenesis in eNOS$^{-/-}$ mice. The role of eNOS in angiogenesis was investigated in vivo by implanting Matrigel in the left ventricular myocardium in anesthetized mice. Three days after implantation, Matrigel was surrounded by fibrosis and was easily identified in the myocardium. Massive in vivo angiogenesis was observed with aneurysm-like structures in wild-type mice (Fig. 5*A*). However, there were only small isolated vessels formed in Matrigel in eNOS$^{-/-}$ mice (Fig. 5*B*). The area of capillaries and aneurysm-like structures penetrating the Matrigel plug was quantified in relation to the total Matrigel area. The percent vessel-like areas were significantly decreased in eNOS$^{-/-}$ mice compared with wild-type mice (Fig. 5*C*; n = 7 mice/group, $P < 0.01$).

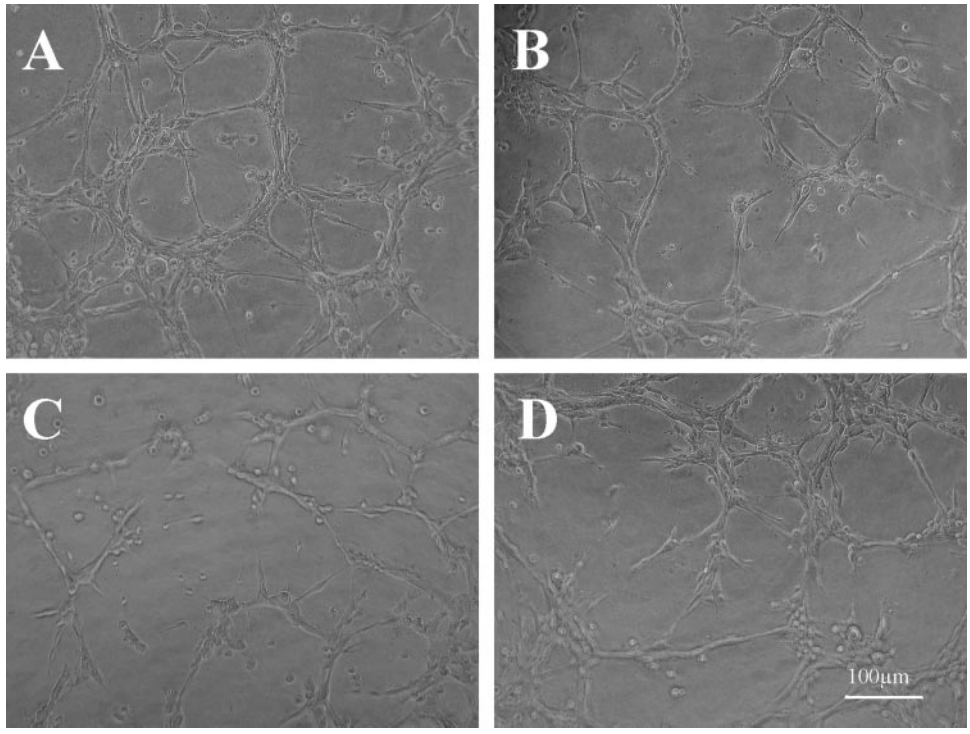

Fig. 3. Representative images of angiogenesis in cardiac endothelial cells from eNOS$^{-/-}$ and wild-type mice. Primary cultures of cardiac endothelial cells from adult mice were seeded onto 96-well plates coated with Matrigel. Images were taken at 4 h. *A*: wild-type control. *B*: N^G-nitro-L-arginine methyl ester (L-NAME) treatment decreased angiogenesis in cardiac endothelial cells from wild-type mice. *C*: eNOS$^{-/-}$ control. *D*: treatment with the NO donor diethylenetriamine (DETA)-NO restored angiogenesis in cardiac endothelial cells from eNOS$^{-/-}$ mice.

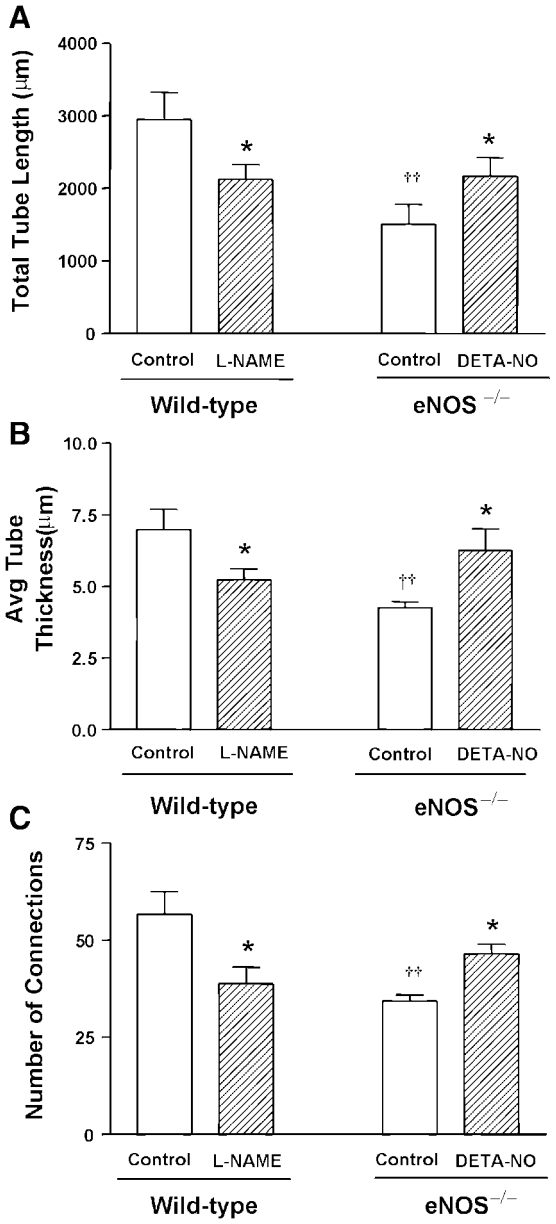

Fig. 4. Quantification of tube formation in cardiac endothelial cells. Cardiac endothelial cells from eNOS$^{-/-}$ mice showed a significant reduction in total tube length (*A*), average (Avg) tube thickness (*B*), and the number of connections (*C*) compared with wild-type mice. L-NAME (500 μM) inhibited tube formation in wild-type mice. DETA-NO (2 μM) restored tube formation in eNOS$^{-/-}$ mice (n = 5 mice/group). *$P < 0.05$ vs. wild-type mice; ††$P < 0.01$ vs. the control group.

Endogenous expression of VEGF in myocardium. To determine whether impaired angiogenesis in eNOS$^{-/-}$ mice is associated with a reduction in endogenous VEGF expression, VEGF mRNA expression in the left ventricular myocardium was analyzed by RT-PCR in both neonatal and adult mice. VEGF mRNA expression in neonatal ventricular myocardium was significantly decreased in eNOS$^{-/-}$ mice compared with wild-type mice (n = 4 mice/group, $P < 0.01$; Fig. 6). However, VEGF mRNA expression in the adult ventricular myocardium was similar between wild-type and eNOS$^{-/-}$

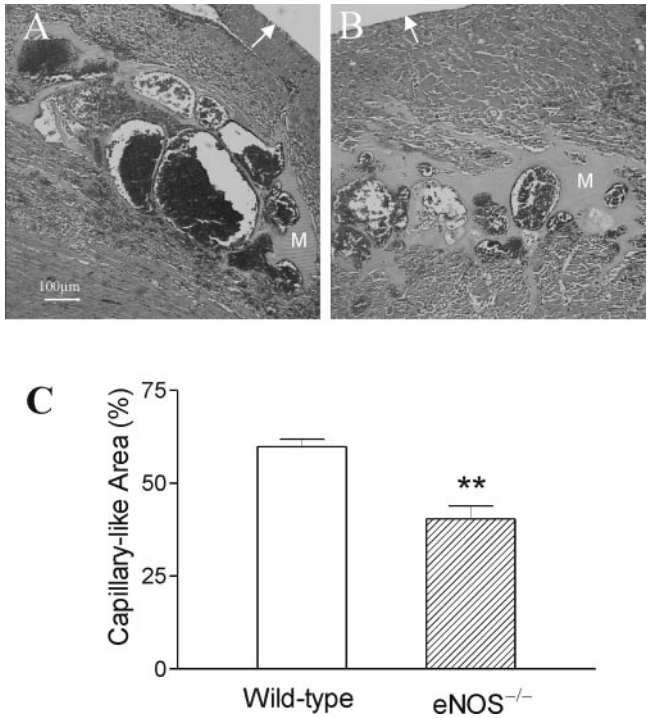

Fig. 5. In vivo myocardial angiogenesis in adult eNOS$^{-/-}$ and wild-type mice. Matrigel (M) was implanted into the LV myocardium for 72 h. *A*: massive angiogenesis with aneurysm-like structures in wild-type mice. *B*: small cannulated isolated vessels in eNOS$^{-/-}$ mice. *C*: quantitative analysis of angiogenesis. The area of capillary-like structures penetrating the Matrigel plug was quantified. Results were expressed as the percentage of the vessel area in the total Matrigel area (n = 7 mice/group). Arrows indicate epicardium. **$P <$ 0.01.

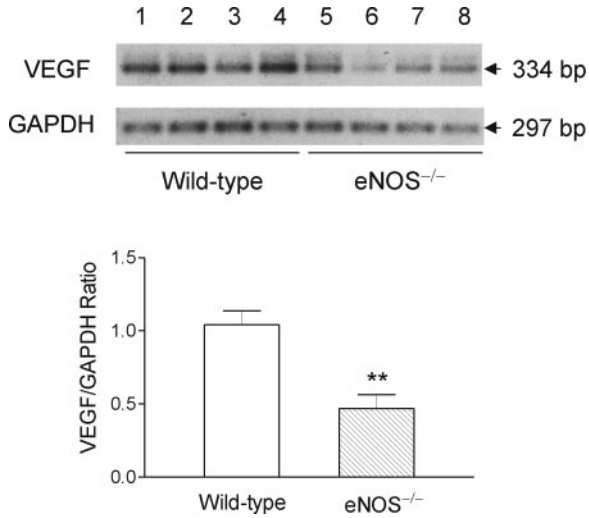

Fig. 6. Expression of vascular endothelial growth factor (VEGF) in the neonatal mouse myocardium. RT-PCR products of VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were electrophoresed through a 2% agarose gel (*top*) and quantified by densitometry (*bottom*). VEGF mRNA expression in the LV myocardium detected by RT-PCR was significantly decreased in eNOS$^{-/-}$ mice compared with wild-type mice (n = 4 mice/group). Each lane represents a separate neonatal mouse. **$P < 0.01$.

mice (VEGF-to-GAPDH optical density ratio: 10.5 ± 0.4 vs. 9.4 ± 1.3, P = NS, n = 3 mice/group).

DISCUSSION

The major finding of this study is that a deficiency in eNOS resulted in significant impairment of myocardial capillary development associated with decreased VEGF expression in the neonatal mouse myocardium. Both in vitro and in vivo myocardial angiogenesis were significantly reduced in eNOS$^{-/-}$ mice. Furthermore, in vitro tube formation was significantly inhibited by L-NAME in wild-type mice, whereas decreased tube formation in eNOS$^{-/-}$ mice was restored by DETA-NO. Taken together, the present study indicates that a deficiency in eNOS decreases VEGF expression and results in impairment of myocardial angiogenesis. NO production from eNOS plays an important role in myocardial angiogenesis.

The morphology of endothelial cells and myocardial capillaries gradually matures during late gestation, and by the early postnatal period there is dramatic growth of the coronary vascular bed (25). In rats, the aggregate capillary length in the ventricular myocardium doubles in the first 11 days postnatal, suggesting that the postnatal period is critical for myocardial capillary development (30). Interestingly, eNOS expression is closely related to myocardial capillary development. Myocardial eNOS expression is gradually increased during late gestation, and by birth there is extensive eNOS expression in myocardial blood vessels and the endocardium, a pattern that is similar to the adult heart (32). In the present study, we demonstrated that myocardial capillary densities were significantly decreased in neonatal eNOS$^{-/-}$ mice, indicating an important role of eNOS in myocardial capillary development. It is possible that decreased myocardial capillary densities induce myocardial ischemia, which may be responsible for the cardiac dysfunction and high mortality in neonatal eNOS$^{-/-}$ mice (9).

A variety of models have been used for study of angiogenesis in vitro and in vivo. In the present study, cardiac endothelial cells were used for in vitro tube formation on Matrigel. We established for the first time an intramyocardial Matrigel model in adult mice to assess in vivo myocardial angiogenesis. The Matrigel could be implanted safely into the mouse left ventricular wall. The unique benefit of this model versus the subcutaneous Matrigel model is that angiogenesis in Matrigel is formed by cardiac endothelial cells in myocardial milieu (4). The Matrigel used in our assay is known to contain an array of growth factors, including endothelial growth factors, platelet-derived growth factor, insulin growth factor 1, transforming growth factor-β, etc. This permitted a direct analysis of the requirement of eNOS in growth factor-stimulated angiogenesis.

NO has been identified as a downstream mediator of various growth factors initiating the angiogenic signaling cascade in endothelial cells (2, 23), and eNOS is the predominant NOS isoform in VEGF-induced angiogenesis in vivo (11). In ischemic limb and wound repair models, eNOS$^{-/-}$ mice showed significantly reduced angiogenesis (17, 20). Inhibition of eNOS by L-NAME attenuated endothelial cell migration, one of the key events for angiogenesis (21). The present study further extended the effects of NO on angiogenesis to the heart. In vivo angiogenesis in Matrigel implanted in the left ventricular myocardium was markedly decreased in eNOS$^{-/-}$ mice. In our in vitro angiogenesis assay, a primary culture of cardiac endothelial cells was seeded on Matrigel to avoid possible loss of eNOS after passages of the endothelial cells in wild-type mice (1). In vitro tube formation from cardiac endothelial cells was significantly decreased in eNOS$^{-/-}$ mice. Total tube length, average tube thickness, and the number of connections were inhibited by L-NAME in wild-type mice and restored by DETA-NO in eNOS$^{-/-}$ mice. The results demonstrated that myocardial angiogenesis is NO dependent, and NO produced by eNOS plays an important role in myocardial angiogenesis.

Studies have shown that VEGF mRNA is strongly expressed in the myocardium, and coronary capillary growth is dependent on VEGF during the prenatal and early postnatal period (30, 31). Deficiency of $VEGF_{164}$ and $VEGF_{188}$ impairs myocardial angiogenesis and induces ischemic cardiomyopathy (5). Partial VEGF inhibition achieved by inducible gene targeting leads to impaired organ development and increased mortality in mice (12). Increased NO production from eNOS induces VEGF expression in vascular smooth muscle cells (7). However, it is not known whether a deficiency of eNOS alters VEGF expression in neonatal hearts. In the present study, we demonstrated that VEGF expression was significantly decreased in neonatal eNOS$^{-/-}$ mice, which was consistent with a decrease in capillary densities. The result suggests that lack of eNOS decreases VEGF expression and may contribute to impaired myocardial capillary development in neonatal eNOS$^{-/-}$ mice.

The potential mechanism underlying the attenuation of VEGF expression in the neonatal eNOS$^{-/-}$ heart is not completely understood. It has been shown that there is a positive feedback between NO and VEGF. NO is not only a downstream mediator of VEGF-induced endothelial cell proliferation and migration but also an upstream promoter of VEGF expression (15). It has been demonstrated that NO increases the transcriptional activity of the VEGF promotor in vascular smooth muscle cells (15) and skeletal muscle (3). In addition, NO prolonged the half-life of VEGF mRNA (6). It is possible that lack of NO production in eNOS$^{-/-}$ mice abrogates the positive feedback mechanism between NO and VEGF and causes a decrease in VEGF expression.

In adult eNOS$^{-/-}$ mice, however, capillary densities were not altered. Our results are consistent with a recent report (16) that demonstrated similar myocardial capillary densities in cardiac and skeletal muscles between adult eNOS$^{-/-}$ and wild-type mice. We did not detect any significant changes of VEGF expression in the myocardium of adult eNOS$^{-/-}$ mice either. These

data agree with a previous report (20) that showed that VEGF expression in skeletal muscle was not altered in adult eNOS$^{-/-}$ mice compared with wild-type mice (20). The mechanism related to well-developed capillary densities in adult eNOS$^{-/-}$ mice is not clear. It is possible that proangiogenic factors are upregulated and compensated for the loss of eNOS function and promote postnatal development of myocardial capillaries in those surviving eNOS$^{-/-}$ mice. Lack of eNOS may induce postnatally an increase in some angiogenic factors, such as angiotensin (22) and prostaglandins (13). Whether these factors are involved in the myocardial capillary development in adult eNOS$^{-/-}$ mice requires further investigation.

In summary, neonatal mice deficient in eNOS showed decreased VEGF expression and impaired capillary development in the myocardium. Both in vitro and in vivo myocardial angiogenesis were decreased in eNOS$^{-/-}$ mice. Our results demonstrated an important role of eNOS in myocardial capillary development and angiogenesis. Decreased myocardial angiogenesis may represent an important mechanism in the myocardial apoptosis, heart failure, and high mortality we recently observed in neonatal eNOS$^{-/-}$ mice (9).

We thank Keith D. Hutcheson for technical assistance in histological preparations.

This study was supported by Canadian Institutes of Health Research (CIHR) Grant MT-14653 and Heart and Stroke Foundation of Ontario Grant T4045 (to Q. Feng). Q. Feng was supported by a Research Career Award from the Rx&D Health Research Foundation and CIHR. X. Zhao was a postdoctoral fellow from Department of Cardiology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, People's Republic of China.

REFERENCES

1. **Ando H, Kubin T, Schaper W, and Schaper J.** Cardiac microvascular endothelial cells express α-smooth muscle actin and show low NOS III activity. *Am J Physiol Heart Circ Physiol* 276: H1755–H1768, 1999.
2. **Babaei S, Teichert-Kuliszewska K, Monge JC, Mohamed F, Bendeck MP, and Stewart DJ.** Role of nitric oxide in the angiogenic response in vitro to basic fibroblast growth factor. *Circ Res* 82: 1007–1015, 1998.
3. **Benoit H, Jordan M, Wagner H, and Wagner PD.** Effect of NO, vasodilator prostaglandins, and adenosine on skeletal muscle angiogenic growth factor gene expression. *J Appl Physiol* 86: 1513–1518, 1999.
4. **Bussolati B, Dunk C, Grohman M, Kontos CD, Mason J, and Ahmed A.** Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. *Am J Pathol* 159: 993–1008, 2001.
5. **Carmeliet P, Ng YS, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, Ehler E, Kakkar VV, Stalmans I, Mattot V, Perriard JC, Dewerchin M, Flameng W, Nagy A, Lupu F, Moons L, Collen D, D'Amore PA, and Shima DT.** Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms $VEGF_{164}$ and $VEGF_{188}$. *Nat Med* 5: 495–502, 1999.
6. **Chin K, Kurashima Y, Ogura T, Tajiri H, Yoshida S, and Esumi H.** Induction of vascular endothelial growth factor by nitric oxide in human glioblastoma and hepatocellular carcinoma cells. *Oncogene* 15: 437–442, 1997.
7. **Dulak J, Jozkowicz A, Dembinska-Kiec A, Guevara I, Zdzienicka A, Zmudzinska-Grochot D, Florek I, Wojtowicz A, Szuba A, and Cooke JP.** Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 20: 659–666, 2000.
8. **Feng Q, Lu X, Jones DL, Shen J, and Arnold JMO.** Increased inducible nitric oxide synthase expression contributes to myocardial dysfunction and higher mortality after myocardial infarction in mice. *Circulation* 104: 700–704, 2001.
9. **Feng Q, Song W, Lu X, Hamilton JA, Lei M, Peng T, and Yee SP.** Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. *Circulation* 106: 873–879, 2002.
10. **Ferrara N and Davis-Smyth T.** The biology of vascular endothelial growth factor. *Endocr Rev* 18: 4–25, 1997.
11. **Fukumura D, Gohongi T, Kadambi A, Izumi Y, Ang J, Yun CO, Buerk DG, Huang PL, and Jain RK.** Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc Natl Acad Sci USA* 98: 2604–2609, 2001.
12. **Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, and Ferrara N.** VEGF is required for growth and survival in neonatal mice. *Development* 126: 1149–1159, 1999.
13. **Godecke A, Decking UK, Ding Z, Hirchenhain J, Bidmon HJ, Godecke S, and Schrader J.** Coronary hemodynamics in endothelial NO synthase knockout mice. *Circ Res* 82: 186–194, 1998.
14. **Hefler LA, Reyes CA, O'Brien WE, and Gregg AR.** Perinatal development of endothelial nitric oxide synthase-deficient mice. *Biol Reprod* 64: 666–673, 2001.
15. **Jozkowicz A, Cooke JP, Guevara I, Huk I, Funovics P, Pachinger O, Weidinger F, and Dulak J.** Genetic augmentation of nitric oxide synthase increases the vascular generation of VEGF. *Cardiovasc Res* 51: 773–783, 2001.
16. **Kubis N, Besnard S, Silvestre JS, Feletou M, Huang PL, Levy BI, and Tedgui A.** Decreased arteriolar density in endothelial nitric oxide synthase knockout mice is due to hypertension, not to the constitutive defect in endothelial nitric oxide synthase enzyme. *J Hypertens* 20: 273–280, 2002.
17. **Lee PC, Salyapongse AN, Bragdon GA, Shears LL, 2nd Watkins SC, Edington HD, and Billiar TR.** Impaired wound healing and angiogenesis in eNOS-deficient mice. *Am J Physiol Heart Circ Physiol* 277: H1600–H1608, 1999.
18. **Lee TC, Zhao YD, Courtman DW, and Stewart DJ.** Abnormal aortic valve development in mice lacking endothelial nitric oxide synthase. *Circulation* 101: 2345–2348, 2000.
19. **Matsunaga T, Warltier DC, Weihrauch DW, Moniz M, Tessmer J, and Chilian WM.** Ischemia-induced coronary collateral growth is dependent on vascular endothelial growth factor and nitric oxide. *Circulation* 102: 3098–3103, 2000.
20. **Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, and Isner JM.** Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101: 2567–2578, 1998.
21. **Murohara T, Witzenbichler B, Spyridopoulos I, Asahara T, Ding B, Sullivan A, Losordo DW, and Isner JM.** Role of endothelial nitric oxide synthase in endothelial cell migration. *Arterioscler Thromb Vasc Biol* 19: 1156–1161, 1999.
22. **Otani A, Takagi H, Suzuma K, and Honda Y.** Angiotensin II potentiates vascular endothelial growth factor-induced angiogenic activity in retinal microcapillary endothelial cells. *Circ Res* 82: 619–628, 1998.
23. **Papapetropoulos A, Garcia-Cardena G, Madri JA, and Sessa WC.** Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 100: 3131–3139, 1997.
24. **Passaniti A, Taylor RM, Pili R, Guo Y, Long PV, Haney JA, Pauly RR, Grant DS, and Martin GR.** A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest* 67: 519–528, 1992.
25. **Porter GA and Bankston PW.** Myocardial capillaries in the fetal and the neonatal rat: a morphometric analysis of the maturing myocardial capillary bed. *Am J Anat* 179: 108–115, 1987.
26. **Risau W.** Mechanisms of angiogenesis. *Nature* 386: 671–674, 1997.

27. **Rui T, Cepinskas G, Feng Q, Ho YS, and Kvietys PR.** Cardiac myocytes exposed to anoxia-reoxygenation promote neutrophil transendothelial migration. *Am J Physiol Heart Circ Physiol* 281: H440–H447, 2001.
28. **Tabibiazar R and Rockson SG.** Angiogenesis and the ischaemic heart. *Eur Heart J* 22: 903–819, 2001.
29. **Tomanek RJ, Lotun K, Clark EB, Suvarna PR, and Hu N.** VEGF and bFGF stimulate myocardial vascularization in embryonic chick. *Am J Physiol Heart Circ Physiol* 274: H1620–H1626, 1998.
30. **Tomanek RJ, Sandra A, Zheng W, Brock T, Bjercke RJ, and Holifield JS.** Vascular endothelial growth factor and basic fibroblast growth factor differentially modulate early postnatal coronary angiogenesis. *Circ Res* 88: 1135–1141, 2001.
31. **Tomanek RJ, Zheng W, Peters KG, Lin P, Holifield JS, and Suvarna PR.** Multiple growth factors regulate coronary embryonic vasculogenesis. *Dev Dyn* 221: 265–273, 2001.
32. **Ursell PC and Mayes M.** Endothelial isoform of nitric oxide synthase in rat heart increases during development. *Anat Rec* 246: 465–472, 1996.