Lack of endothelial nitric oxide synthase decreases cardiomyocyte proliferation and delays cardiac maturation

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Lack of endothelial nitric oxide synthase decreases cardiomyocyte proliferation and delays cardiac maturation. Am J Physiol Cell Physiol 291: C1240–C1246, 2006. First published July 5, 2006; doi:10.1152/ajpcell.00092.2006.—We recently demonstrated that deficiency in endothelial nitric oxide synthase (eNOS) results in congenital septal defects and postnatal heart failure. The aim of this study was to investigate the role of eNOS in cardiomyocyte proliferation and maturation during postnatal development. Cultured eNOS knockout (eNOS–/–) cardiomyocytes displayed fewer cells and lower bromodeoxyuridine (Brdu) incorporation in vitro compared with wild-type (WT) cardiomyocytes (P < 0.05). Treatment with the nitric oxide (NO) donor diethylenetriamine NONOate increased BrdU incorporation and cell counts in eNOS–/– cardiomyocytes (P < 0.05). Inhibition of nitric oxide synthase activity using Nω-nitro-L-arginine methyl ester decreased the level of BrdU incorporation and cell counts in WT cardiomyocytes (P < 0.05). Vascular endothelial growth factor (VEGF) increased the level of BrdU incorporation in cultured WT cardiomyocytes in a dose- and time-dependent manner (P < 0.05). Conversely, VEGF did not alter BrdU incorporation in eNOS–/– cardiomyocytes (P = not significant). Furthermore, deficiency in eNOS significantly decreased BrdU labeling indices in neonatal hearts in vivo. Although WT hearts displayed a rapid decrease in atrial natriuretic peptide (ANP) expression in the first week of neonatal life, ANP expression in eNOS–/– hearts remain elevated. Our study demonstrated that NO production from eNOS is necessary for postnatal cardiomyocyte proliferation and maturation, suggesting that eNOS plays an important role during postnatal heart development. Proli...
10^5) in polystyrene, nonpyrogenic 96-well culture plates (Becton Dickinson, Franklin Lakes, NJ) precoated with 1% gelatin. Cells were incubated in 5% CO₂ at 37°C. To determine cardiomyocyte purity, cells were seeded on glass slides and cultured for 72 h. Cells were then fixed in 20% acetic acid-80% methanol, stained with a primary monoclonal antibody against cardiac α-actinin, and followed by a rhodamine-conjugated secondary antibody. Nuclei were stained with Hoechst. Cells were examined under a Zeiss fluorescence microscope (×630). Cardiomyocytes stained by α-actinin antibody showed characteristic striation.

**Drug treatment.** After 24 h in culture medium supplemented with 10% FBS, cardiomyocytes were either starved in serum-free medium or maintained with serum for different drug treatment. Cardiomyocytes were then stimulated with either basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) at concentrations ranging from 10 to 100 ng/ml. To assess the relationship between NO and cardiomyocyte proliferation, WT cardiomyocytes were treated with a nonselective NOS inhibitor molecule arginine methyl ester (L-NAME), and eNOS−/− cardiomyocytes were treated with the NO donor diethylenetriamine NO (DETA-NO). All drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO) unless specified.

**Cardiomyocyte cell count.** The level of proliferation in cultured cardiomyocytes was first determined by cell counts. After cardiomyocytes were cultured for 24, 48, 72, and 96 h, cells were detached with 50 μl of 0.5% trypsin for 10–15 min. Cells were counted using a hemocytometer and an automated NucleoCounter (New Brunswick Scientific).

**Cardiomyocyte DNA synthesis in vitro.** Cellular proliferation was also quantified via BrdU (Roche Molecular Diagnostics, Montreal, Quebec, Canada) incorporation into the DNA of proliferating cardiomyocytes isolated from both eNOS−/− and WT mice. BrdU incorporation into actively dividing cardiomyocytes was determined by immunohosting according to the manufacturer’s instructions. Briefly, cells were grown for 18, 42, 66, and 90 h. At the respective growth periods, cells were labeled with the pyrimidine analog BrdU at a concentration of 10 μM for a period of 6 h. After BrdU incubation, cells were washed with PBS buffer solution (pH 7.4) and fixed with 200 μl of precooled 70% ethanol in HCl (vol/vol) at a final concentration of 0.5 M HCl. Subsequently, cells were washed three times with PBS and incubated with 100 μl exomucilase III (0.4 U/μl; MBI Fermentas, Burlington, Ontario, Canada) at 37°C for 30 min. Next, the cells were treated with the primary monoclonal antibody anti-BrdU conjugated with peroxidase (Roche Molecular Diagnostics) at 37°C for 30 min. Cells were incubated with the peroxidase substrate (2,2’-azino-bis,3-ethylbenzthiazoline-6-sulfonic acid; ABTS) for 10 min at room temperature to allow color development. The absorbance of the samples was measured at 405 nm in a Bio-Rad microtiter plate reader (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

**Cardiomyocyte DNA synthesis in vivo.** As an index of DNA synthesis, BrdU incorporation was measured in vivo using a BrdU-labeling and staining kit (Roche Molecular Diagnostics). BrdU (50 mg/kg) was administered to neonatal mice subcutaneously. After (6 h), animals were killed, and hearts and small intestine (positive control) were obtained. Frozen tissue sections (10 μm) were stained for BrdU incorporation.

Briefly, cryosections were fixed in 70% ethanol in 2.4 mM HCl for 20 min at −20°C. Endogenous peroxidase activity was inhibited by incubating tissue sections in 0.3% H₂O₂-H₂O (vol/vol). Tissue sections were treated with protease K (20 μg/ml; Gibco-BRL, Burlington, Ontario, Canada) to expose the antigens. Sections were incubated with mouse anti-BrdU (1:10) for 1 h at 37°C in a humidified atmosphere. After incubation with the primary antibody, the slides were washed in PBS buffer solution (pH 7.4) three times and incubated with the secondary antibody, biotinylated horse anti-mouse IgG (1:50; Vectorstain ABC Kit; Vector Laboratories, Burlingame, CA), for 30 min at room temperature. Tissue sections were then washed with PBS buffer solution (pH 7.4) and incubated with an avidin-biotin-peroxidase amplification system (Vector Laboratories) for 30 min at room temperature. BrdU incorporation into proliferating cells was detected after incubation with the peroxidase substrate 3,3’-diaminobenzidine tetrahydrochloride at a concentration of 1 mg/ml (wt/vol) in PBS for 10 min at room temperature. Tissue sections were subsequently counterstained in Harris modified hematoxylin (Fischer Scientific, Fairlawn, NJ). Labeled nuclei cells with striation were quantified in 10 independent fields by microscopic analysis (Leitz) using a ×50 objective with the BrdU-labeling index expressed as the ratio of BrdU positive cells to total nuclei.

**Nitrite measurement.** The formation of nitrite in culture medium was used as an indicator of NO production by neonatal cardiomyocytes. Culture medium was concentrated for five times before nitrite levels were measured by Griess reaction as we described previously (24, 27). The nitrite concentration was expressed as nanomoles per milligram cell protein.

**RNA isolation and RT-PCR.** Total RNA was isolated from neonatal hearts of WT and eNOS−/− mice with TRIzol reagent (GIBCO-BRL) and subsequently reverse transcribed into first-strand cDNA using the Moloney murine leukemia virus RT system (MOP-64395; GIBCO-BRL). The mRNA expression of ANP and α-MHC was determined by semiquantitative RT-PCR. Oligonucleotide primers were synthesized by Gibco-BRL. The cDNA was amplified by PCR using a programmable thermal cycler. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to ensure that an equal amount of RNA was present in all samples. Target gene accession numbers, reaction primer sequences, cycle numbers, and annealing temperatures are summarized in Table 1. PCR samples were then electrophoresed, separated on a 1.2% agarose gel containing ethidium bromide, and visualized under ultraviolet light to detect the expression of ANP and α-MHC. Samples were analyzed via computer densitometry, and all densitometric data were standardized to GAPDH mRNA.

**Statistical analysis.** Data are expressed as means ± SE. Differences between WT and eNOS−/− groups were compared using unpaired Student’s t-test. For multigroup comparisons, ANOVA followed by the Student-Newman-Keuls test was performed (SigmaStat version 1.0).
RESULTS

Cellular proliferation in neonatal cardiomyocytes in vitro. Purity of cardiomyocyte after 72 h of cell culture was determined by immunocytochemical staining using a monoclonal antibody specific for cardiac muscle sarcomeric protein α-actinin, which stained Z-lines and showed typical striation of cardiomyocytes. Purity of cardiomyocytes from WT and eNOS−/− cultures was 94.6 ± 0.8 and 93.4 ± 2.1%, respectively [n = 3/group, P = not significant (NS)]. Proliferation of cultured neonatal cardiomyocytes was determined by cell count and BrdU incorporation. Results obtained through cell cultured neonatal cardiomyocytes was determined by cell count revealed that ~80% of the 100,000 cardiomyocytes initially seeded survived after 24 h in the cell culture model system employed (Fig. 1A). WT cardiomyocytes showed an increase in cell number and the level of BrdU incorporation over a 96-h time course (Fig. 1, A and B). Interestingly, cardiomyocytes deficient in eNOS displayed significantly fewer cells and lower BrdU incorporation compared with the WT cardiomyocytes after 72 and 96 h in culture (P < 0.05; Fig. 1, A and B).

NO production was measured in the culture medium. Nitrite levels were significantly decreased in eNOS−/− compared with WT cardiomyocytes (9.8 ± 0.8 vs. 14 ± 1.0 μmol/mg protein, P < 0.01). Treatment with NO donor DETA-NO (2 μM) increased the nitrite levels of eNOS−/− cardiomyocytes to 19.5 ± 3.5 μmol/mg protein, which was similar to the nitrite levels of WT cardiomyocytes. To demonstrate a causal relationship between NO and cardiomyocyte proliferation, eNOS−/− cardiomyocytes were treated with DETA-NO (2 μM). DETA-NO treatment increased the absolute number of cardiomyocytes and the level of BrdU incorporation in eNOS−/− cardiomyocytes at 72 and 96 h (P < 0.05; Fig. 1, A and B). In addition, treatment with the NOS inhibitor L-NAME (500 μM) significantly decreased cell number and BrdU incorporation at 72 and 96 h in WT cardiomyocytes (P < 0.05; Fig. 1, A and B) to levels similar in eNOS−/− cardiomyocytes. Treatment with L-NAME for 96 h had no significant effects on cell number in eNOS−/− cardiomyocytes (90,938 ± 534 vs. 88,125 ± 1,420 cells/well for control and L-NAME treatment, respectively; n = 4 independent experiments; P = NS).

To investigate if the effects of NO on cardiomyocyte proliferation were mediated by cGMP, WT cardiomyocytes were treated with the selective guanylate cyclase inhibitor ODQ (100 μM) for 96 h. ODQ significantly decreased cell number in WT cardiomyocytes (129,875 ± 1,120 vs. 89,542 ± 1,142 cells/well; n = 4 independent experiments, P < 0.01). These results suggest that the effects of NO on cardiomyocyte proliferation are mediated by cGMP.

Effect of VEGF on the proliferation of eNOS−/− and WT cardiomyocytes. The ability of VEGF to stimulate cardiomyocyte proliferation was evaluated over time. WT cardiomyocytes treated with VEGF (30 ng/ml) displayed increased levels of BrdU incorporation over the unstimulated controls at 24, 48, 72, and 96 h (P < 0.05; Fig. 2A). On the basis of this time course, 48 h of VEGF treatment were chosen for the subsequent experiment. The effects of VEGF on BrdU incorporation were examined with and without serum. In the presence of serum, WT cardiomyocytes treated with VEGF displayed increased levels of BrdU incorporation in a dose-dependent manner, with maximal stimulation observed at a concentration of 100 ng/ml (P < 0.05; Fig. 2B). Conversely, VEGF did not significantly alter the level of BrdU incorporation in eNOS−/− cardiomyocytes (P = NS; Fig. 2B). The effects of VEGF (30 and 100 ng/ml) on BrdU incorporation were significantly decreased in eNOS−/− compared with WT cardiomyocytes (P < 0.05; Fig. 2B). Interestingly, in the absence of serum, VEGF did not significantly alter the level of BrdU incorporation in either WT or eNOS−/− cardiomyocytes (P = NS; Fig. 2B).
Effect of bFGF on the proliferation of eNOS\(^{-/-}\) and WT cardiomyocytes. bFGF has been demonstrated to be a potent stimulator of neonatal cardiomyocyte proliferation in vitro (14); however, the corresponding relationship with NO remains unclear. Consequently, the effect of bFGF on the proliferation of eNOS\(^{-/-}\) and WT cardiomyocytes was investigated in the present study. A dose-response profile of the effects of bFGF was performed in WT and eNOS\(^{-/-}\) cardiomyocytes at concentrations of 10, 30, and 100 ng/ml. In the presence of serum, bFGF significantly enhanced levels of BrdU incorporation over the respective controls in both groups (\(P < 0.05\); Fig. 3). However, no significant difference in the level of BrdU incorporation was observed between WT or eNOS\(^{-/-}\) cardiomyocytes. In the absence of serum, bFGF did not significantly alter BrdU incorporation in either group (\(P = \text{NS}\); Fig. 3).

Relationship between bFGF and VEGF on cardiomyocyte proliferation. To investigate a possible synergistic or additive interaction between the mitogenic agents bFGF and VEGF, cultured WT cardiomyocytes were stimulated with bFGF and VEGF concurrently in the presence of serum. Treatment of WT cardiomyocytes with bFGF (10 ng/ml) or VEGF (10 ng/ml) significantly increased the level of BrdU incorporation observed over the control group (\(P < 0.05\); Fig. 4). Furthermore, concurrent treatment with both bFGF and VEGF significantly enhanced the level of BrdU incorporation over that seen in cardiomyocytes treated with either factor individually (\(P < 0.05\); Fig. 4). Despite the increase in the level of BrdU incorporation, no additive or synergistic effect was observed between VEGF and bFGF.

Cellular proliferation in neonatal mouse hearts in vivo. The level of proliferation occurring in vivo in the myocardium of neonatal WT and eNOS\(^{-/-}\) mice was monitored during the 1st wk of postnatal life and quantified by the BrdU labeling index. The time course investigation of the level of proliferation demonstrated that the BrdU labeling index in the WT myocardium was 9.9% at postnatal day 1, declined at postnatal day 3, and subsequently increased to 14.7% at postnatal day 5 (Fig. 5). Cardiomyocytes in the myocardium of eNOS\(^{-/-}\) mice...
displayed significantly attenuated levels of BrdU incorporation at postnatal day 1 and day 5 compared with their WT counterparts (*P < 0.05; Fig. 5).

Expression of ANP and α-MHC mRNA in neonatal hearts. The mRNA of ANP from neonatal hearts of WT and eNOS^−/− mice was determined by semiquantitative RT-PCR using GAPDH as a loading control. PCR products of ANP and GAPDH obtained from both WT and eNOS^−/− hearts are shown in Fig. 6A. The densitometric ANP-to-GAPDH ratios were significantly decreased in postnatal day 7 hearts of WT mice compared with postnatal day 1 hearts (P < 0.05; Fig. 6B). Cardiac α-MHC mRNA expression was also evaluated in the hearts of WT and eNOS^−/− mice by semiquantitative RT-PCR. PCR products generated from both WT and eNOS^−/− neonatal mouse hearts are shown in Fig. 6C. The densitometric α-MHC-to-GAPDH ratios were significantly decreased at postnatal days 5 and 7 in eNOS^−/− compared with WT mice (P < 0.05; Fig. 6D).

Fig. 5. In vivo BrdU labeling in the myocardium of neonatal WT and eNOS^−/− mice. Neonatal animals were treated with BrdU (50 mg/kg sc) for 6 h. Immunohistological staining for BrdU was performed on frozen sections of heart tissues. The myocardium of eNOS^−/− mice displayed significantly decreased BrdU incorporation compared with WT controls at postnatal day 1 (P1) and postnatal day 5 (P5). P3, postnatal day 3. *P < 0.05 vs. WT; n, no. of animals at each time point/group.

Fig. 6. Expression of atrial natriuretic peptide (ANP) and α-myosin heavy chain (α-MHC) mRNA in the myocardium of WT and eNOS^−/− neonatal hearts. Hearts were obtained from eNOS^−/− and WT mice at P1 to postnatal day 7 (P7). Expression of ANP and α-MHC mRNA was determined by RT-PCR. A: representative amplifications of ANP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in WT and eNOS^−/− hearts. B: ANP-to-GAPDH optical density (OD) ratio was significantly decreased at P7 compared with P1 in WT hearts. C: α-MHC and GAPDH amplification in WT and eNOS^−/− neonatal hearts. D: α-MHC-to-GAPDH OD ratio was not significantly altered from P1 to P7 in either WT or eNOS^−/− neonatal hearts. However, α-MHC-to-GAPDH OD ratio was significantly decreased at P5 and P7 in eNOS^−/− compared with WT hearts. P < 0.05 vs. WT at P1 (†) and vs. corresponding WT (‡); n, no. of animals at each time point/group.

DISCUSSION

The results from our study demonstrate for the first time the importance of NO derived from eNOS in modulating proliferation and maturation of cardiomyocytes in mice. Deficiency in eNOS resulted in significantly fewer cell numbers and lower levels of BrdU incorporation in cultured mouse cardiomyocytes, as well as decreased BrdU labeling indexes in the neonatal hearts of eNOS^−/− mice in vivo. Furthermore, eNOS^−/− hearts displayed prolonged expression of ANP. These results suggest that eNOS contributes to proliferation and maturation of cardiomyocytes during early postnatal heart development.

Recent studies from our group and others support a role for eNOS in heart development (7, 18). However, mechanisms by which eNOS affects cardiac development are not fully understood. Interestingly, eNOS has been shown to regulate proliferation of several cell types, including endothelial cells and vascular smooth muscle cells (9, 22). However, the role of NO in cardiomyocyte proliferation has not previously been investigated. In the present study, cultured eNOS^−/− cardiomyocytes displayed significantly decreased cell numbers and lower BrdU incorporation compared with WT cardiomyocytes. Treatment with the NO donor DETA-NO increased proliferation of cultured eNOS^−/− cardiomyocytes. In our study, DETA-NO did not fully restore eNOS^−/− cardiomyocyte proliferation to the WT level. This suggests that genetic deletion of eNOS gene may result in changes that also affect cardiomyocyte proliferation and cannot be corrected by an exogenous NO donor. On the other hand, inhibition of NOS activity using L-NAME in WT cardiomyocytes inhibited proliferation similar to that seen in the eNOS^−/− population. Furthermore, in vivo BrdU labeling indexes in the neonatal myocardium at postnatal days 1 and 5 were significantly decreased in eNOS^−/− com-
pared with WT mice. These data suggest that eNOS modulates cardiomyocyte proliferation in vitro and in vivo.

Normal cardiac development is dependent on the coordinated expression and interaction of numerous growth factors and hormones. The expression of VEGF, for example, is tightly controlled such that either conditional deletion or over-expression of VEGF leads to cardiovascular malformation and embryonic lethality (8, 11). Although several growth factors have shown to exert mitogenic effects on cultured cardiomyocytes (16, 20), the role of NO in mediating this process is still not clear. In the present study, the effects of exogenous VEGF and bFGF on myocyte proliferation were explored. VEGF stimulated the proliferation of WT cardiomyocytes in a dose- and time-dependent manner. However, this effect was blunted in eNOS−/− cardiomyocytes, suggesting that VEGF-induced proliferation occurs through an NO-dependent mechanism. However, bFGF stimulated proliferation of WT and eNOS−/− cardiomyocytes to a similar extent, indicating that bFGF-induced proliferation occurs via an NO-independent pathway. Studies have shown that VEGF and bFGF have a synergistic effect on angiogenesis (25). To test whether there is any interaction between VEGF and bFGF on cardiomyocyte proliferation, WT cardiomyocytes were concurrently treated with VEGF and bFGF. Our results showed that VEGF and bFGF did not have a synergistic effect on cardiomyocyte proliferation.

ANP and MHC are differentiation markers expressed during heart development. Myocardial ANP expression decreases as the neonatal heart is maturing, and its expression eventually ceases in the mature ventricular myocardium (13, 14). MHC expression is also a differentiation marker for cardiac maturation. During neonatal development, there is a switch of MHC isoforms expressed in the myocardium. Although both α-MHC and β-MHC are expressed in the neonatal myocardium, the predominant MHC isoform is α-MHC in the mature myocardium (21, 29). In the present study, ANP mRNA expression progressively decreased after birth in the WT hearts. However, ANP expression remained elevated in eNOS−/− hearts during the 1st wk of neonatal development. In addition, myocardial α-MHC expression was significantly decreased at postnatal days 5 and 7 in eNOS−/− compared with WT mice. These results suggest that myocardial maturation of eNOS−/− mice is delayed.

The present study suggests that NO produced from eNOS is important for cardiomyocyte proliferation and maturation during neonatal heart development. NO has also been shown to promote cardiomyogenesis (4, 15). These effects of NO may in part contribute to the congenital heart defects and heart failure we previously reported in eNOS−/− mice (7). Deficiency in eNOS also leads to limb defects in mice (10). Interestingly, TBX5, a T-box-containing transcriptional factor expressed during early heart development, stimulates cardiomyogenesis and cardiomyocyte proliferation (12). Mutation of TBX5 in humans and deficiency in TBX5 in mice result in congenital heart and limb defects, the Holt-Oram syndrome (2, 3, 5). It is not known if there is any interaction between NO and TBX5. However, these studies and the present data do support the notion that factors regulating cardiomyogenesis and cardiomyocyte proliferation are important to fetal and neonatal heart development.

In summary, the present study demonstrated that proliferation is decreased in cultured eNOS−/− cardiomyocytes and in the hearts of neonatal eNOS−/− mice in vivo. The effect of VEGF on cardiomyocyte proliferation is NO dependent, whereas bFGF stimulates cardiomyocyte proliferation via NO-independent mechanisms. Furthermore, myocardial ANP expression is prolonged and α-MHC expression is decreased in eNOS−/− mice, suggesting that cardiac maturation is delayed. We conclude that NO derived from eNOS plays an important role in cardiomyocyte proliferation and maturation during early neonatal heart development.

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