Nitric oxide synthase in cardiac sarcoplasmic reticulum

KAI Y. XU†, DAVID L. HUSO‡, TED M. DAWSON§, DAVID S. BREDT¶, AND LEWIS C. BECKER*†

*Department of Medicine, Division of Cardiology and ‡Division of Comparative Medicine, Johns Hopkins Medical Institutions, Baltimore, MD 21224; §Department of Neurology and Neuroscience, Johns Hopkins Medical Institutions, Baltimore, MD 21287; and ¶Department of Physiology, University of California School of Medicine, San Francisco, CA 94143

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved November 6, 1998 (received for review July 1, 1998)

ABSTRACT NO is a free radical that modulates heart function and metabolism. We report that a neuronal-type NO synthase (NOS) is located on cardiac sarcoplasmic reticulum (SR) membrane vesicles and that endogenous NO produced by SR-associated NOS inhibits SR Ca2+ uptake. Ca2+-dependent biochemical conversion of L-arginine to L-citrulline was observed from isolated rabbit cardiac SR vesicles in the presence of NO substrates and cofactors. Endogenous NO was generated from the vesicles and detected by electron paramagnetic resonance spin-trapping measurements. Immunoelectron microscopy demonstrated labeling of cardiac SR vesicles by using anti-neuronal NOS (nNOS), but not anti-endothelial NOS (eNOS) or anti-inducible NOS (iNOS) antibodies, whereas skeletal muscle SR vesicles had no nNOS immunoreactivity. The nNOS immunoreactivity also displayed a pattern consistent with SR localization in confocal micrographs of sections of human myocardium. Western blotting demonstrated that cardiac SR NOS is larger than brain NOS (160 vs. 155 kDa). No immunodetection was observed in cardiac SR vesicles from NOS knockout mice or with an anti-nNOS antibody, suggesting the possibility of a new nNOS-type isoform. Ca2+ uptake by cardiac SR vesicles, catalyzed by Ca2+-ATPase, was inhibited by NO produced endogenously from cardiac SR NOS, and 7-nitroindazole, a selective nNOS inhibitor, completely prevented this inhibition. These results suggest that a cardiac muscle nNOS isoform is located on SR of cardiac myocytes, where it may respond to intracellular Ca2+ concentration and modulate SR Ca2+ ion active transport in the heart.

METHODS

Isolation of Cardiac SR Vesicles. Cardiac muscle SR vesicles were prepared from hearts of New Zealand White rabbits or from knockout and wild-type, C57BL6 mice (Charles River Breeding Laboratories) according to the method of Chu et al. (11) with modifications similar to those described previously (12). Animal care was in accordance with institutional guidelines. The final vesicles were resuspended in 10 mM Tris-HCl and 0.29 M sucrose buffer, pH 7.4, and stored at −70°C. Protein concentration was determined by the method of Lowry et al. (13).

Determination of NOS Activity. NOS activity was measured by monitoring the conversion of L-[3H]arginine to L-[3H]citrulline (14). For routine assays, 0.1 mg of vesicles and 10 μl of 100 mM L-[3H]arginine were added to 40 μl of buffer containing 50 mM Tris (pH 7.4), 1 mM NADPH, 0.2 mM CaCl2, 0.1 μM calmodulin (CaM), 2 μM flavin adenine dinucleotide (FAD), 2 μM flavin mononucleotide (FMN), and 3 μM of tetrahydrobiopterin (BH4). After incubation for 30 min at 23°C, the reaction was stopped with 0.4 μl of 50 mM Hepes (pH 5.5) and 2 mM EGTA, and then applied to a 1-ml column of Dowex AG50WX-8 (Na+), which was eluted with 2 ml of water. The L-[3H]citrulline, being ionically neutral at pH 5.5, flowed through the column completely. The NOS activity was then quantitated by determining the radioactivity in the flow through.

Electron Paramagnetic Resonance (EPR) Measurements. Spin trapping measurements of NO radicals were performed in a flat cell at room temperature. The final concentrations of N-methyl-d-glucamine dithiocarbamate (MGD), Fe2+ vesicles, Ca2+, CaM, NADPH, FAD, FMN, BH4, L-arginine, d-arginine, and superoxide dismutase (SOD) were 5 mM, 1 mM, 0.6 mg/ml, 0.2 mM, 0.1 μM, 1 mM, 2 μM, 2 μM, 3 μM, 2 mM, 2 mM, and 300 units/ml, respectively. The EPR spectra was recorded immediately after the spin trap solution was infused by using an IBM-Bruker ER 300 spectrometer operating at X-band with a transverse magnetic 110 cavity. The spectrometer settings were as follows: modulation frequency, 100 kHz; modulation amplitude, 1 G; scan time 1.0 min; microwave power, 80 mW; and microwave frequency, 9.7 GHz.

Western Blotting. The Opti-4CN Goat Anti-Mouse Detection Kit was used for the Western Blotting experiments (Bio-Rad). For purposes of nNOS immunodetection, three different antibodies were used. The anti-nNOSα antibody was raised against an N-terminal fusion protein encoded by exon 2 of nNOS and recognizes nNOSα (15). The anti-nNOSα

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: NOS, NO synthase; iNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; SR, sarcoplasmic reticulum; EPR, electron paramagnetic resonance; RyR, ryanodine receptor; CaM, calmodulin; BH4, tetrahydrobiopterin; MGD, N-methyl-d-glucamine dithiocarbamate; SOD, superoxide dismutase; TRIM, 1-(2-trifluoromethylphenyl)imidazole; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine.

†To whom reprint requests should be addressed. e-mail: kxu@welchlink.welch.jhu.edu and lbecker@welchlink.welch.jhu.edu.
antibodies were diluted in PBS 10% horse serum. Secondary antibodies used at 1:500 were donkey anti-mouse IgG or anti-rabbit IgG from Jackson Immunoresearch. Anti-nNOS [MA3–910], anti-Na,K-ATPase (MA3–928), and anti-phospholamban (MA3–922) for double labeling. All of these antibodies were conjugated to 12-nm colloidal gold diluted 1:40 in PBS. After 30 min of incubation at room temperature, the grids were washed with PBS and then incubated for 30 min with a secondary antibody (donkey anti-mouse IgG or anti-rabbit IgG from Jackson Immunoresearch) conjugated to 12-nm colloidal gold diluted 1:40 in PBS. After the labeling, all grids were contrasted with lead citrate and examined on a Zeiss 10A transmission electron microscope operating at 80 kV. Double immunogold labeling was performed by incubating grids with a second primary antibody (anti-nNOS) diluted 1:250 in PBS after the first molecule (Ca2+-ATPase, Na+,K+-ATPase, or phospholamban) was labeled with primary and secondary antibodies. After a 30-min incubation, the grids were rinsed with PBS and then incubated for 30 min with a second secondary antibody conjugated to 12-nm colloidal gold diluted 1:40 in PBS. The grids were then washed, stained, dried, and viewed as described above.

**Immunofluorescent Labeling**. The label was performed after modifications (17). A biopsy of human ventricular muscle from an explanted heart was frozen in liquid nitrogen-cooled isopentane, embedded in OCT, and stored at −80°C until sectioned at 6 μm. The sections were dried onto glass slides, rehydrated, and blocked with PBS 10% horse serum. Primary mAbs to cardiac SR Ca2+-ATPase (SERCA2, Affinity BioReagents), RyR, Ca2+-ATPase, or phospholamban was labeled with primary and secondary antibodies. After a 30-min incubation, the grids were rinsed with PBS and then incubated for 30 min with a secondary antibody conjugated to 12-nm colloidal gold diluted 1:40 in PBS. The grids were then washed, stained, dried, and viewed as described above.

**Immunofluorescent Labeling**. The labeling was performed as described with modifications (17). A biopsy of human ventricular muscle from an explanted heart was frozen in liquid nitrogen-cooled isopentane, embedded in OCT, and stored at −80°C until sectioned at 6 μm. The sections were dried onto glass slides, rehydrated, and blocked with PBS 10% horse serum. Primary mAbs to cardiac SR Ca2+-ATPase (SERCA2, Affinity BioReagents), RyR, Ca2+-ATPase, or phospholamban was labeled with primary and secondary antibodies. After a 30-min incubation, the grids were rinsed with PBS and then incubated for 30 min with a secondary antibody conjugated to 12-nm colloidal gold diluted 1:40 in PBS. The grids were then washed, stained, dried, and viewed as described above.

**Immunofluorescent Labeling**. The labeling was performed as described with modifications (17). A biopsy of human ventricular muscle from an explanted heart was frozen in liquid nitrogen-cooled isopentane, embedded in OCT, and stored at −80°C until sectioned at 6 μm. The sections were dried onto glass slides, rehydrated, and blocked with PBS 10% horse serum. Primary mAbs to cardiac SR Ca2+-ATPase (SERCA2, Affinity BioReagents), RyR, Ca2+-ATPase, or phospholamban was labeled with primary and secondary antibodies. After a 30-min incubation, the grids were rinsed with PBS and then incubated for 30 min with a secondary antibody conjugated to 12-nm colloidal gold diluted 1:40 in PBS. The grids were then washed, stained, dried, and viewed as described above.

**Immunofluorescent Labeling**. The labeling was performed as described with modifications (17). A biopsy of human ventricular muscle from an explanted heart was frozen in liquid nitrogen-cooled isopentane, embedded in OCT, and stored at −80°C until sectioned at 6 μm. The sections were dried onto glass slides, rehydrated, and blocked with PBS 10% horse serum. Primary mAbs to cardiac SR Ca2+-ATPase (SERCA2, Affinity BioReagents), RyR, Ca2+-ATPase, or phospholamban was labeled with primary and secondary antibodies. After a 30-min incubation, the grids were rinsed with PBS and then incubated for 30 min with a secondary antibody conjugated to 12-nm colloidal gold diluted 1:40 in PBS. The grids were then washed, stained, dried, and viewed as described above.

**Immunofluorescent Labeling**. The labeling was performed as described with modifications (17). A biopsy of human ventricular muscle from an explanted heart was frozen in liquid nitrogen-cooled isopentane, embedded in OCT, and stored at −80°C until sectioned at 6 μm. The sections were dried onto glass slides, rehydrated, and blocked with PBS 10% horse serum. Primary mAbs to cardiac SR Ca2+-ATPase (SERCA2, Affinity BioReagents), RyR, Ca2+-ATPase, or phospholamban was labeled with primary and secondary antibodies. After a 30-min incubation, the grids were rinsed with PBS and then incubated for 30 min with a secondary antibody conjugated to 12-nm colloidal gold diluted 1:40 in PBS. The grids were then washed, stained, dried, and viewed as described above.
D-arginine in the presence of vesicles and cofactors (Fig. 1a, lane D), demonstrating that cardiac SR NOS activity is Ca$^{2+}$-dependent. It has been reported that 7-NI and 1-(2-trifluoromethylphenyl)imidazole (TRIM) are potent, selective inhibitors of nNOS (18, 19), with an IC_{50} for 7-NI of 0.71μM for rat nNOS and of 28.2μM for TRIM for mouse nNOS (20). TRIM is a poor inhibitor of bovine aortic endothelial NOS with an IC_{50} of 1057.5μM (20). The complete inactivation of cardiac SR NOS activity under our experimental conditions required 10μM 7-NI (Fig. 1a, lane F) or 60μM TRIM (Fig. 1a, lane G). These results suggest that cardiac SR NOS is an nNOS-type NOS and is not eNOS. The potent iNOS inhibitor, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, 10 nM), completely inhibited iNOS (Fig. 1a, lane I), but only 15% inhibition of cardiac SR NOS was seen at the same concentration (Fig. 1a, lane H), indicating that cardiac SR NOS is not iNOS.

It has been reported that both skeletal and cardiac muscle express nNOS_{μ} isoform (4). To evaluate whether cardiac SR NOS is nNOS_{μ} protein, Western blot experiments were performed by using an anti-nNOS_{μ} antibody. nNOS_{μ} was detected in whole heart homogenates. Cardiac SR NOS also was detected by anti-nNOS and anti-nNOSα antibodies in both isolated rabbit and mouse cardiac SR vesicles, but not in the nNOS knockout mouse cardiac SR vesicles (Fig. 1b). In contrast, no detection occurred when the same vesicles were incubated with anti-nNOS_{μ} antibody, suggesting that cardiac SR NOS is not an nNOS_{μ} isoform. The results also show that cardiac SR NOS was larger than brain NOS (160 vs. 155 kDa). Taken together, these results suggest the possibility of a potential nNOS isoform associated with the cardiac SR membrane.

We next examined whether NO' is produced by cardiac SR vesicles by using the NO'-specific spin trap Fe^{2+} complex, Fe(MGD)$_2$ (21). EPR spectroscopy is a technique capable of directly measuring molecules with unpaired electron spin such as NO'. The EPR spin-trapping measurements confirmed that NO' was generated endogenously from isolated SR vesicles in the presence of NOS substrates and cofactors (Fig. 2D, a triplet spectrum). This endogenous NO' production was inhibited by the nNOS selective inhibitor, 7-NI (Fig. 2E) and could not be supported by d-arginine (Fig. 2F). The results further demonstrate functional NOS in cardiac SR vesicles. To ensure the specificity of endogenous NO' generation, SOD was present in all of the reaction samples, except Fig. 2A and B, to prevent superoxide generation from BH$_4$ autoxidation (22) or any other source. NO' radicals were not detected in a mixture of SOD (300 units/ml), Ca$^{2+}$ (0.2 mM), L-arginine (2 mM), NADPH (1 mM), FAD (1 μM), FMN (1 μM), CaM (0.1 μM), and BH$_4$ (3 μM) as shown in Fig. 2C. Moreover, neither the Fe(MGD)$_2$ spin-trapping reagent alone (Fig. 2A) nor with SOD (Fig. 2B) showed any detectable NO' radical signal.

Immunofluorescence localization of cardiac SR NOS was next performed in human ventricular myocardial sections to determine how its subcellular location compared with the restricted sarcosomal membrane localization pattern that has been reported for skeletal muscle (1–4, 7). By using a mAb (Transduction Laboratories) specific to nNOS, the results revealed a combination of a longitudinal and transverse linear intracellular pattern in the heart muscle (Fig. 3a,A) consistent with what has been described for antigens localized to the longitudinal and junctional SR (23, 24). For comparison, we stained sections with mAbs specific for the cardiac Ca$^{2+}$-

These results show that 7-NI sensitive, endogenous NO' (a triplet spectrum) is generated by the isolated cardiac SR vesicles. The data are presented from one of four similar independent experiments in each case.
ATPase (SERCA2) and the RyR, both of which have previously been shown to be localized to the longitudinal and junctional SR (25, 26). The SR antigens, RyR (Fig. 3aC) and SERCA2 (Fig. 3aD), had a similar immunofluorescent pattern to nNOS (Fig. 3aA). In contrast, Na\(^+\),K\(^+\)-ATPase, which is exclusively a sarcolemmal antigen, had a completely different pattern of immunofluorescent staining (Fig. 3aB). Control sections stained with a mAb to an irrelevant viral protein displayed only weak background fluorescence (Fig. 3aE).

To confirm the immunological identity and localization of the NOS isoform responsible for endogenous NOS activity in cardiac SR vesicles, immunogold labeling and electron microscopy were performed. Anti-nNOS immunoreactivity was broadly distributed on isolated cardiac SR membrane vesicles.
Fig. 4. Effect of endogenous NO on cardiac SR 45Ca uptake under various conditions. Vesicles in the absence of NOS substrates, cofactors, and SOD (A); -SOD + thapsigargin (B) (Tg, a specific inhibitor of Ca2+-ATPase); vesicles + SOD (C); + SOD + Tg (D); condition C in the presence of NOS substrates and cofactors for 30 min (E), or for 60 min (F) (30 min at 23°C and 30 min at 37°C); 60-min incubation (same as F) in the presence of the NOS inhibitor 7-NI (10 μM) (G); d-arginine replaced l-arginine in condition F (H). Data represent the mean ± SD from six independent experiments by using 0.6 mg/ml cardiac SR vesicles. The concentration of SOD was 300 units/ml in all reaction mixtures. 45Ca uptake is inhibited by endogenously produced NO. The results suggest that cardiac NOS regulates SR 45Ca uptake by directly modifying Ca2+-ATPase function through endogenous NO.

DISCUSSION

The studies reported here show immunocytochemically and biochemically that a neuronal-type NOS is associated with the cardiac SR membrane. The formation of both l-citrulline and NO2 radicals from isolated cardiac SR vesicles provides strong evidence that a functional NOS exists on cardiac SR vesicles. Studies have shown that nNOS is located in the endoplasmic reticulum in the rabbit cerebellum (27), but in skeletal muscle, it is restricted to the sarcolemma by a PDZ protein targeting sequence (nNOSμ) (1–4, 7, 28). Our finding that cardiac SR NOS is slightly larger than brain NOS (160 vs. 155 kDa) suggests that cardiac SR NOS may be a new splice or posttranslationally modified variant of nNOS. Although cardiac SR NOS migrates at the same molecular mass as NOSμ, it probably is not an NOS2 isoform because it was not detected by a specific NOS2a antibody. Our findings indicate that it is an NOS isoform as cardiac SR NOS is readily detected by selective NOS antibodies and this detection is completely absent in nNOS null mice. Our results show by the immunofluorescence that anti-nNOS immunoreactivity was in a pattern consistent with SR localization in human heart sections, rather than being restricted to the cardiac sarcolemma. Immunogold colocalization studies confirmed nNOS immunoreactivity on cardiac SR vesicles, but not on skeletal muscle SR vesicles, suggesting that the cardiac nNOS isoform may not be the same as that previously described for skeletal muscle (1–4).

It has been reported recently by Zahradnikova et al. (10) that eNOS is associated with cardiac SR membrane. However, we could not detect eNOS in our isolated cardiac SR vesicles by either immunogold labeling (Fig. 3) or Western blotting (data not shown). We further tested whether caveola membranes are associated with our SR vesicles because eNOS is targeted to caveolae (29–31). Western blot analysis revealed that caveolae membrane protein, caveolin 3, which is expressed in smooth, skeletal and cardiac muscle, was found in wild-type mice or rabbit whole heart homogenates, but was not detected in our isolated rabbit and mice cardiac SR vesicles or in nNOS knockout-mice cardiac SR vesicles (data not shown). The apparent discrepancy regarding the presence of eNOS on cardiac SR vesicles may possibly be explained by the presence of caveola membranes or sarcolemma in the SR vesicle preparations used in the study by Zahradnikova et al. (10). The mechanism underlying the association of nNOS-like NOS with cardiac SR membranes is still unknown. Whether the binding of NOS to cardiac SR is mediated by electrostatic interaction (32–34) remains to be answered.

SR Ca2+ stores are central to the dynamic cytosolic Ca2+ fluxes that occur during contraction/relaxation cycling in the beating heart. NO has been found to be an important regulator of muscle contractility (1–8, 25, 26). It has been reported that NO inhibits both the RyR calcium release channel and force production in skeletal muscle and attenuates cardiac myocyte contraction (35–37). Our results provide new evidence that NO also can directly down-regulate the SR Ca2+-ATPase and impair the SR calcium transport system (Fig. 4). NO-induced effects on both the SR Ca2+-ATPase and RyR calcium release channel function (37) could result in a marked alterations in SR Ca2+ release, cytosolic Ca2+ concentration, and heart muscle contraction. The inhibitory effect of NO is likely to be further influenced by increases in intracellular calcium as demonstrated previously under ischemic conditions (38). The detailed biochemical mechanism underlying the regulatory effect of NO on SR Ca2+ active transport is not clear. However, NO can regulate SR Ca2+-ATPase and the RyR by reacting with regulatory thiolis of these proteins (39–41) or through a new pathway involving S-nitrosogluthathione (42).

The molecular basis for the modulation of heart function by NO is only partly understood. Our results not only localize a possible new isoform of the neuronal-type NOS on cardiac SR, but also provide evidence for a molecular interaction between NO and cardiac SR Ca2+-ATPase that may serve to regulate cardiac SR Ca2+ active transport. More detailed investigations
of how this NOS isoform is associated with the cardiac SR membrane and how NO affects SR Ca\textsuperscript{2+} transport in cardiomyocytes, should increase our understanding of the bioregulatory role of NO in cellular Ca\textsuperscript{2+} homeostasis and cardiac muscle contraction during health and disease.

We are grateful to Dr. Ralph Hruban for providing human heart tissue, Dr. Charles J. Lowenstein for help with the NOS activity assay, and Drs. Peter L. Pedersen, David Proud, Anne Kagey-Sobotka, Perianam Kuppusamy, Jay Zweier, and James Shem for useful suggestions and discussions. We thank Daniel Guastella for assistance with confocal microscopy. This work was supported by Grants HL 52175 (to K.Y.X.), HL33360 (to D.L.H.), and NS34822 (to D.S.B.) from the National Institute of Neurological Institute and NS35693 (to D.L.H.), NS33277 (to T.M.D.), and P50 HL52315 (to L.C.B.), and T32 HL07783-01 (to L.C.B.), and NS34822 (to D.S.B.) from the National Institute of Neurological Disorders and Stroke.