Arginase modulates myocardial contractility by a nitric oxide synthase 1-dependent mechanism

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Cardiac myocytes contain two constitutive NO synthase (NOS) isoforms with distinct spatial locations, which allows for isoform-specific regulation. One regulatory mechanism for NOS is substrate (L-arginine) bioavailability. We tested the hypothesis that arginase (Arg), which metabolizes L-arginine, constrains NOS activity in the cardiac myocyte in an isoform-specific manner. Arg activity was detected in both rat heart homogenates and isolated myocytes. Although both Arg I and II mRNA and protein were present in whole heart, Arg II alone was found in isolated myocytes. Arg inhibition with *S*-(2-boronoethyl)-L-cysteine (BEC) augmented Ca^{2+}-dependent NOS activity and NO production in myocytes, which did not depend on extracellular L-arginine. Arg II coimmunoprecipited with NOS1 but not NOS3. Isolation of myocyte mitochondrial fractions in combination with immuno-electron microscopy demonstrates that Arg II is confined primarily to the mitochondria. Because NOS1 positively modulates myocardial contractility, we determined whether Arg inhibition would increase basal myocardial contractility. Consistent with our hypothesis, Arg inhibition increased basal contractility in isolated myocytes by a NOS-dependent mechanism. Both the Arg inhibitors *N*-hydroxy-*nor*-L-arginine and BEC dose-dependently increased basal contractility in rat myocytes, which was inhibited by both nonspecific and NOS1-specific NOS inhibitors N^G-nitro-L-arginine methyl ester and *S*-methyl-L-thiocitrulline, respectively. Also, BEC increased contractility in isolated myocytes from WT and NOS3 but not NOS1 knockout mice. We conclude that mitochondrial Arg II negatively regulates NOS1 activity, most likely by limiting substrate availability in its microdomain. These findings have implications for therapy in pathophysiologic states such as aging and heart failure in which myocardial NO signaling is disrupted.

mitochondria | L-arginine pools | spatial confinement

Recent evidence has clearly demonstrated the critical role of NO synthase (NOS) isoforms in the spatial confinement of NO signaling in the heart (1–3). Specifically, in the sarcoplasmic reticulum (SR), NOS1 colocalizes with the ryanodine receptor (RYR), and activation of NOS1 positively modulates cardiac contractility. Also, NOS1 deficiency leads to an increase in xanthine oxidase-dependent reactive-oxygen species activity, which dramatically depresses myocardial contractile function (4). In contrast, the NOS3 isoform coupled to the β_3 adrenergic receptor (AR), inhibits L-type Ca^{2+} channels and, thus, inhibits β-AR-mediated increases in myocardial contractility (5).

NO signaling may be mediated by a soluble guanylyl cyclase-dependent increase in cGMP (6) or cGMP-independent nitrosylation of a broad spectrum of effector proteins (7). An emerging body of evidence indicates that the balance between NO and O_2^- regulates the NO/redox balance, thus determining the nitrosylation of proteins and their resultant physiologic or pathophysiologic effects (8).

Although the activity and abundance of enzymes important in the regulation and dysregulation of the NO/redox balance in physiological and pathophysiological conditions (for example, heart failure) have been characterized (9), the mechanisms that regulate the pivotal NOS enzyme substrate L-arginine remain poorly understood. An emerging paradigm in NO biology indicates that arginase (Arg), an enzyme that also uses L-arginine as a substrate, reciprocally regulates NOS activity. This phenomenon has been demonstrated for both constitutive (10, 11) and inducible (12) NOS isoforms, where Arg constrains (and, thus, regulates) NOS activity. Also, up-regulation of Arg has been shown to contribute to the pathophysiology of disease processes in which NO signaling is dysregulated [for example, the endothelial dysfunction of aging (10), hypertension (13, 14), and atherosclerosis (15), the erectile dysfunction of diabetes (16), and reactive airways disease in asthma (17–19)].

However, the role of Arg in modulating NOS activity in the heart is unknown. Thus, we tested the hypothesis that Arg modulates myocardial contractility in a spatially confined manner. Here, we demonstrate that Arg II is expressed in cardiac myocytes, is confined primarily to the mitochondria, reciprocally regulates NOS, and offsets basal myocardial contractility by specifically modulating NOS1 in a spatially confined manner.

Results

Arg Expression and Activity in Cardiac Myocytes. First, we determined whether Arg was expressed in heart tissue and isolated myocytes by Western blot (WB) analysis (rat liver was used as a control for Arg I and kidney as a control for Arg II). Fig. 1*A* shows the expression of Arg II in isolated myocytes. Whereas Arg II is expressed exclusively in the cardiac myocytes, both Arg I and II are found in whole-heart homogenates. These findings most likely reflect the Arg that is present in cell types other than myocytes, such as endothelial cells that have been shown to express Arg I (10, 20). Consistent with the data from WB, immunostaining demonstrated Arg II but not Arg I in isolated myocytes (Fig. 1*A*). To confirm the findings described above, we performed RT-PCR by using mRNA derived from isolated myocytes and whole heart (Fig. 1*A*). Supporting our protein expression data, Arg II mRNA is expressed only in the isolated myocytes, whereas both isoforms are expressed in the whole heart. Next, we determined whether Arg activity was present in the heart and isolated myocytes. Arg activity was detected in cardiac tissue and was inhibitable by the specific Arg inhibitor *S*-(2-boronoethyl)-L-cysteine (BEC) in a dose-dependent manner (Fig. 1*B*). As described, because Arg is expressed and exhibits activity in nonmyocyte cells in the heart (for example, endothelial

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Abbreviations: Arg, arginase; NOS, NO synthase; COX, cytochrome oxidase; SS, sarcomere shortening; L-NAME, N^G-nitro-L-arginine methyl ester; AR, adrenergic receptor; SR, sarcoplasmicreticulum; SERCA, SR Ca^{2+}-ATPase; BEC, *S*-(2-boronoethyl)-L-cysteine; VDAC, voltage-dependent anion channel; Nor-NOHA, *N*-hydroxy-*nor*-L-arginine; WB, Western blot analysis; SMTC, *S*-methyl-L-thiocitrulline; RYR, ryanodine receptor.

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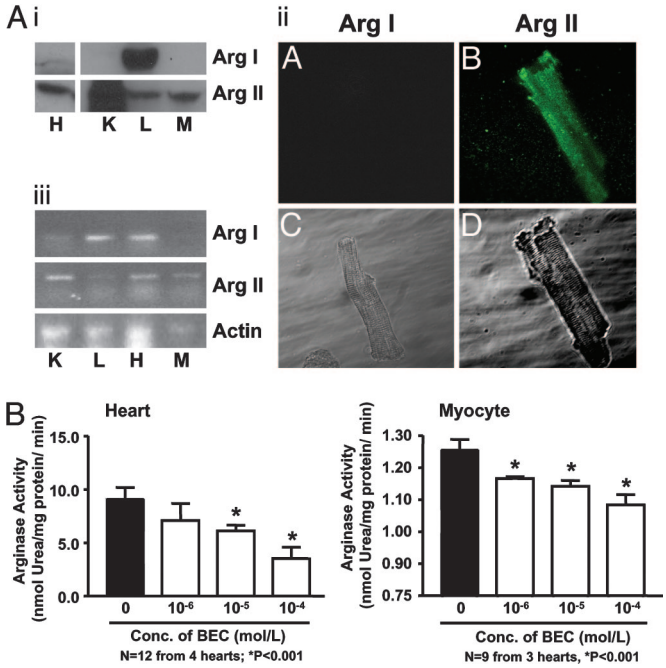

Fig. 1. Arg expression and activity in rat heart and myocytes. (*A*) (*i*) Expression of Arg isoforms in both rat heart (H) and isolated myocyte (M) homogenates by immunoblotting. Although Arg II is confined exclusively to cardiac myocytes, Arg I and II are demonstrated in whole-heart homogenates. Rat liver (L) homogenate is a positive control for Arg I, and rat kidney (K) is a positive control for Arg II. (*ii*) Immunocytochemistry demonstrating Arg II but not Arg I in isolated rat myocytes. Isolated myocytes were fixed and immunofluorescence was detected with Arg II and Cy5-conjugated anti-rabbit Abs. (*iii*) RT-PCR confirming the mRNA expression of Arg I and II in whole heart but Arg II alone in isolated myocytes. (*B*) Arg activity is present in both whole rat heart ($n = 4$) and isolated rat myocytes ($n = 3$). Although Arg activity was significantly higher in the heart than in isolated myocytes, the activity was inhibited in the presence of the specific Arg inhibitor, BEC, in a dose-dependent manner (*, $P < 0.001$ vs. control).

cells), we measured Arg activity in isolated cardiac myocytes. Although Arg activity is lower in myocytes compared with heart tissue, this activity is inhibitable by BEC in a dose-dependent fashion (Fig. 1*B*).

Interaction of Arg and NOS. Next, we determined whether a molecular interaction exists between Arg II and NOS isoforms. Cardiac myocyte protein lysates were coimmunoprecipitated with NOS1- and NOS3-specific Abs, and WB was performed with Arg II Abs. Also, lysates were immunoprecipitated with Arg II Ab, and WB was performed with NOS1 or NOS3 Abs. As shown in Fig. 2*A*, Arg II was detected in lysates that were immunoprecipitated with NOS1 but not NOS3 Abs. Also, NOS1 but not NOS3 was detected in lysates immunoprecipitated with Arg II. This finding is consistent with a specific molecular interaction and/or common or closely adjacent subcellular localization between NOS1 and Arg II.

Next, we determined whether Arg could reciprocally regulate NOS activity. NO production was measured in heart lysates and lysates from isolated cardiac myocytes. BEC-induced inhibition of Arg significantly increased NO production in both the heart (16.7 ± 1 vs. 8.07 μmol per mg of protein; $n = 6$, $P < 0.001$) and isolated myocyte lysates (11.1 ± 2.2 vs. 5.7 ± 1.2 μmol per mg of protein; $n = 6$, $P < 0.001$) (Fig. 2*B*). This observation is consistent with the hypothesis that Arg constrains NOS activity, most likely by limiting substrate availability. Interestingly, the addition of exogenous L-arginine (0.1 mM) alone to the assay buffer did not effect NO production by isolated myocytes. These data support the idea that specific pools of L-arginine are available to NOS isoforms, some of which may not be influenced by extracellular L-arginine (21, 22) (see *Discussion* for more details).

Subcellular Localization of Arg in Cardiac Myocytes. Based on the molecular association between Arg II and NOS, we next sought to determine the subcellular localization of Arg II. NOS1 has been demonstrated to reside in the SR and mitochondria (see ref. 23 for review). In the SR, NOS1 is associated closely with the RYR (3, 24), where it likely regulates its nitrosylation state and, thus, its capacity to release Ca^{2+} (3, 24). Given the tight association between the SR and mitochondria (an association that critically regulates coupling of cardiac excitation and oxidative energy production in the mitochondria) and the fact that Arg II is known to contain a putative leader sequence that targets it to the mitochondria (25, 26), we designed experiments to examine the subcellular location of Arg II within the cardiac myocyte. Mitochondria and crude SR fractions were prepared from rat heart homogenates. As shown in Fig. 3*A*, Arg II is detected in the mitochondrial protein fraction, with a very small amount being present in the cytoplasmic fraction (lactate dehydrogenase as positive control). SR Ca^{2+}-ATPase (SERCA) is also present in proteins prepared from this mitochondrial fraction. The voltage-dependent anion channel (VDAC), which is present only on the outer mitochondrial membrane, was used as our positive control. Because of the difficulty of isolating the mitochondria from the SR by subcellular fractionation, we attempted to determine whether Arg II was confined to the mitochondria or was present in the SR in intact cardiac myocytes. Coimmunoprecipitation of rat heart lysates with Arg II demonstrated a tight association of Arg II with the mitochondrial protein cytochrome oxidase (COX) IV (Fig. 3*B*), implying a predominantly mitochondrial localization of Arg II. To define the spatial location of the Arg II enzyme definitively, we performed immunogold staining and electron microscopy in rat heart tissue. As shown in Fig. 3*C*, Arg II immunogold staining is confined predominantly to the mitochondria within the cardiac myocyte. Also, as shown in Fig. 3*D*, Arg II appears to localize primarily to the periphery of the myocyte mitochondrion, providing direct visual evidence of the Arg II enzyme within the mitochondria at locations that would facilitate close interaction with proteins in the SR membrane.

Effect of Arg–NOS Interaction on Myocardial Contractility. Next, we determined the physiologic effects of Arg on basal myocardial contractility by examining the effect of Arg inhibition on isolated myocyte sarcomere shortening (SS). SS was measured in isolated myocytes in a perfusion chamber before and after the addition of the specific Arg inhibitors BEC or *N*-hydroxy-*nor*-L-arginine (Nor-NOHA) (Fig. 4). Given our observation that Arg II appears to be associated with NOS1, and that NOS1-derived NO accentuates myocardial contractility, we hypothesized that inhibition of Arg would increase basal contractility. Consistent with our hypothesis, BEC increased myocardial contractility in a dose-dependent manner [$\log EC_{50}$, -5.8 ± 0.9; E_{max}, 1.8 ± 0.3 (fold increase)] (Fig. 4*A*). Also, N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM) abolished the increase in contractility that was observed with Arg inhibition (2.1 ± 0.14 vs. 1.1 ± 0.23, for BEC vs. BEC plus L-NAME; $P < 0.001$) such that the E_{max} was similar to baseline (1.1 ± 0.23 vs. 1.0, for BEC + L-NAME vs. baseline; no significant difference). Thus, Arg inhibition exerts its effect by a NOS-dependent mechanism. Also, consistent with our observations, incubation of cardiac myocytes with Nor-NOHA (a pharmacologically distinct specific Arg inhibitor) also caused a dose-dependent increase in basal myocardial contractility ($\log EC_{50}$, -5.8 ± 0.8; E_{max}, 1.98 ± 0.23) (Fig. 4*B*). The EC_{50} values for BEC and Nor-NOHA are consistent with the K_i values of the inhibitors for Arg as described in ref. 27.

Next, we investigated which NOS isoform is constrained by Arg (Fig. 5). *S*-methyl-L-thiocitrulline (SMTC; 10 μM), a specific NOS1 inhibitor, abolished the increase in contractility observed with BEC

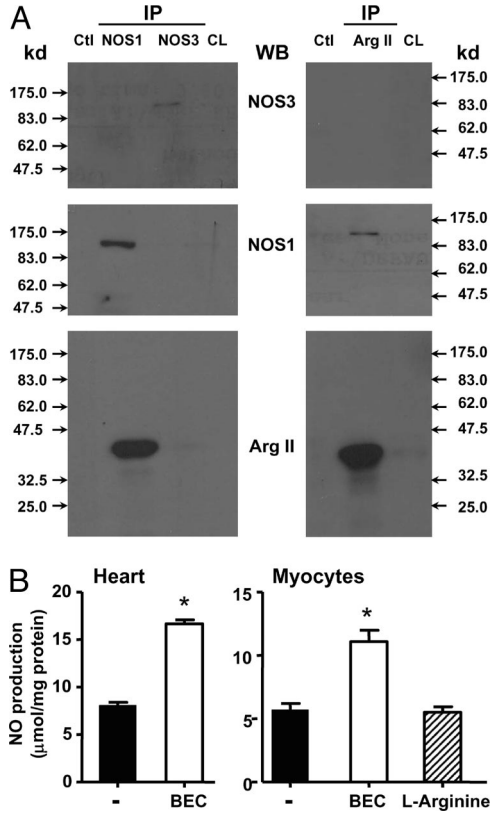

Fig. 2. Interaction of Arg and NOS. (*A*) To determine whether a molecular interaction exists between Arg II and NOS isoforms, cardiac myocyte lysates were immunoprecipitated (IP) with NOS1 or NOS3 Abs and immunoblotted with an Arg II Ab. Also, myocyte lysates were immunoprecipitated with Arg II Ab and immunoblotted with NOS1 and NOS3 Abs. Ctl, negative control; CL, cleared lysate. (*B*) Inhibition of both heart and cardiac myocyte Arg resulted in a significant (≈2-fold) increase in heart and myocyte NO production (*, $P < 0.001$). Addition of exogenous L-arginine (0.1 mM) had no effect on myocyte NO production.

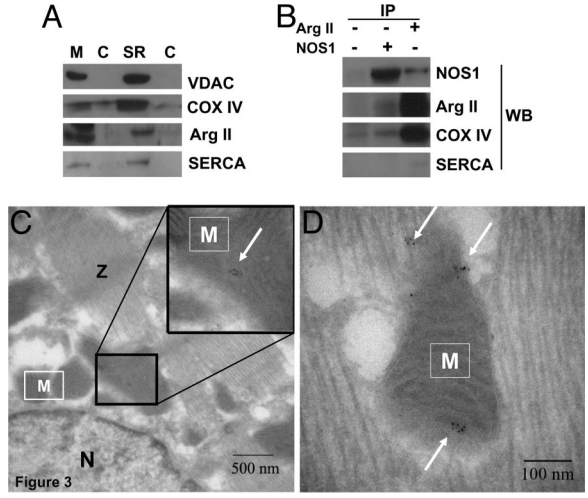

Fig. 3. Subcellular localization of Arg II in cardiac myocytes. (*A*) WB of VDAC, COX IV, Arg II, and SERCA in mitochondrial (M), SR, and cytoplasmic (C) fractions that were prepared from isolated cardiac myocytes. Arg II is localized predominately in the mitochondrial fraction, with some signal in the SR fraction and very little in the cytoplasmic fraction (lactate dehydrogenase as positive control). The detection of Arg II and the mitochondrial proteins VDAC and COX IV in SR fraction is suggestive of the tight association between the mitochondrial and SR compartments. This finding is supported also by the presence of SERCA in the mitochondrial fraction as well as the SR, highlighting the inability to completely separate these two fractions with our current fractionation methods. (*B*) WB of coimmunoprecipitated proteins from rat myocyte lysates by using anti-Arg II and anti-NOS1 Abs. The left lane is the negative control ($Arg\ II^-/NOS1^-$), and the center and right lanes show proteins immunoprecipitated with NOS1 ($Arg\ II^-/NOS1^+$) and Arg II ($Arg\ II^+/NOS1^-$), respectively. Immunoprecipitation of COX IV with Arg II, as shown in the right lane, suggests mitochondrial localization of Arg II. Immunoprecipitation of Arg II and COX IV with NOS1 and NOS1 with Arg II further implies a specific molecular interaction and/or closely adjacent subcellular localization of Arg II in mitochondria and NOS1 in the SR. Immunoelectron microscopy was used to visualize Arg II with Ab-conjugated 6-nm gold beads in rat heart histological sections. (*C*) Trasmission electon micrograph at ×30,000 magnification shows a nucleus (N), *Z*-line of a myofibril (Z), and mitochondria (M) adjacent to a myofibril. The highlighted area in the center of the image is magnified in *Inset* at ×120,000 showing a cluster of gold beads labeling Arg II (white arrow) within a mitochondrion. (*D*) A myocyte mitochondrion (M) at ×120,000 enclosing several clusters of Arg II (white arrows) primarily located at the periphery, consistent with close spatial association with the SR.

(2.06 ± 0.14 vs. 1.24 ± 0.161, for BEC vs. BEC plus SMTC; $P < 0.001$) (Fig. 5). Furthermore, we used WT and NOS1 or NOS3-deficient mice to determine the effect of Arg inhibition on basal contractility. As shown in Fig. 5*B*, BEC caused a dose-dependent increase in basal SS in both WT (E_{max}, 1.97 ± 0.24) and NOS3-deficient (E_{max}, 1.81 ± 0.17) mice. In marked contrast, there was no increase in contractility, as measured by SS, in myocytes from NOS1-deficient mice (E_{max}, 1.11 ± 0.08; $P < 0.001$, vs. NOS3 and WT). Whereas L-NAME alone resulted in a small, but significant, reduction in SS (0.76 ± 0.06 fold change; $n = 3$), L-arginine (0.1 mM) alone had no effect on myocyte contractility (1.1 ± 0.05; $n = 3$, no significant difference). This observation agrees with the findings that exogenous L-arginine has no effect on myocyte NO production. Together, these physiologic data are consistent with the hypothesis that Arg constrains NOS1 activity and, thus, NOS1-dependent myocardial contractility.

Discussion

We have demonstrated that Arg is present predominantly in the mitochondria of cardiac myocytes where it inhibits NOS1 activity, thus regulating NO production and ultimately basal myocardial contractility. These observations provide insights into myocardial NO signaling and its spatial confinement. It appears that not only are the physiologic effects of NO defined by the specific isoform and its microdomain within the cell, but NO is further regulated by the availability of substrate within that enzyme domain. These results demonstrate the complexities of the regulatory mechanisms controlling myocardial contractile function and highlight another protein that exerts a regulatory interaction with NOS1.

Spatial Confinement of NO Signaling in the Heart. Although it has been recognized for over a decade that NOS isoforms are present in the heart, only recently has their functional role in the regulation of E–C coupling been elucidated. It is established that NO modulates the activity of a number of key ion channels and proteins that regulate Ca^{2+} release and thus modulate E–C coupling. Also, NO can either accentuate or attenuate myocardial contractility. This complex and sometimes directionally opposite effect of NO is accomplished by different NOS isoforms being localized to specific cellular microdomains. In this regard, NOS1, localized to the SR (4, 24), is associated with the RYR and SERCA receptors, where it augments Ca^{2+} release in response to frequency (3) and β-AR stimulation (2). In contrast, NOS3 localized to sarcolemmal caveolae (28) negatively regulates L-type Ca^{2+} channels and attenuates the response to β_1-AR activation (29). This effect is mediated by β_3 ARs, which are coupled to the NOS3 isoform (30). These dual and opposing effects of β_3-AR activation are cGMP/guanylyl cyclase-dependent. In contrast, the mechanisms underlying the effects of

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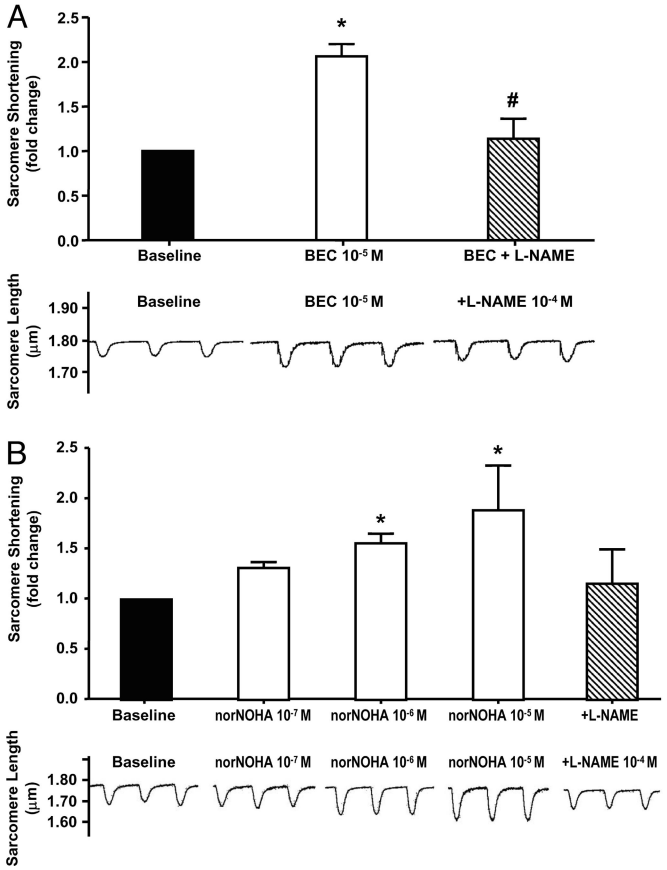

Fig. 4. Effect of Arg inhibition on basal myocardial contractility. (*A*) Isolated rat cardiac myocytes were perfused with Tyrode's solution with or without BEC 10^{-5} M alone or in combination with L-NAME (10^{-4} M). BEC increased contractility (2.1 ± 0.14) as measured by fold change in SS ($n = 8$ cells, $n = 3$ hearts; *, $P < 0.001$). This response was completely inhibited with the non-specific NOS inhibitor, L-NAME (10^{-4} M) ($P < 0.001$). (*B*) Nor-NOHA, doses dependently increased contractility (SS) (1.9 ± 0.45 fold increase; *, $P < 0.05$), the effect of which was specifically inhibited in the presence of L-NAME.

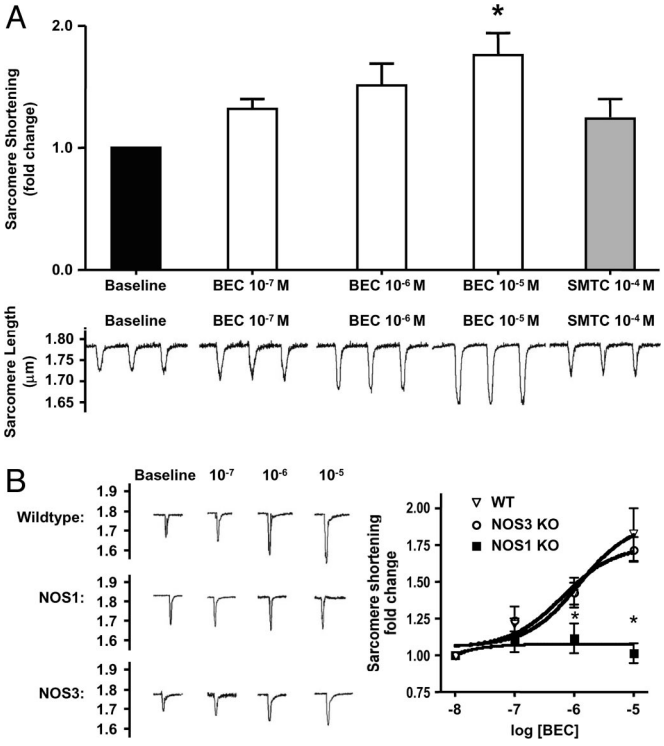

Fig. 5. The effect of Arg inhibition on myocardial contractility is NOS1-isoform-specific. (*A*) BEC dose-dependently increased SS in isolated rat myocytes ($n = 7$, from three hearts; *, $P < 0.01$). This effect was inhibited by the NOS1-specific inhibitor SMTC. (*B*) Isolated myocytes from WT, NOS1, and NOS3 mice were perfused with Tyrode's solution containing increasing doses of BEC. BEC dose-dependently increased SS in both WT and NOS3-deficient mice but had no effect on contractility in NOS1-deficient mice ($n = 11$, from three hearts; no significant difference was determined from baseline; *, $P < 0.001$, vs. WT and NOS3). KO, knockout.

NOS1/NO on SR Ca release are mediated by alterations in nitrosylation of the RYR and possibly the SERCA channels (24, 31). Our observation that Arg interacts with NOS1 and selectively regulates its activity suggests that Arg also has a role in this complex regulatory process.

NO/Redox Balance/Imbalance in Normal and Failing Hearts. Nitrosylation, which is a highly conserved posttranslational mechanism, is recognized to regulate the function of a spectrum of proteins (8). Nitrosylation, the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine, depends on the redox milieu in that region of the protein. The ratio of superoxide/NO production by NOS is an important determinant of the redox milieu. It is established that both skeletal (32), and cardiac (31) RYRs are, in fact, activated by *S*-nitrosylation (33). The cardiac ryanodine isoform, which is *S*-nitrosylated under basal conditions, has been shown to colocalize with NOS1 in the SR (24, 34). NOS1 positively modulates contractility, as demonstrated by depressed force frequency and β-adrenergic inotropic responses in NOS1-deficient mice (2, 3). Together, these data are consistent with the premise that NOS1 modulates the activation of RYRs, perhaps by means of alterations in the redox milieu and levels of RYR nitrosylation. Our result indicating that inhibition of Arg enhances basal myocardial contractility is consistent with this paradigm and suggests that Arg modulates NOS1 and its products, superoxide, and NO. Specifically, the enhanced basal contractility observed with Arg inhibition is abolished in the presence of the specific NOS1 inhibitor SMTC. Also, the response to Arg inhibition is absent in NOS1-deficient mice, but preserved in NOS3-deficient mice.

Many studies have suggested that altered NOS and NO production may contribute to heart failure. However, a coherent hypothesis detailing the role of specific NOS isoforms and the locus of action of NO in heart failure has not yet emerged. Some studies indicate that cytokine-induced NOS2 and NO production cause suppression of myocyte Ca^{2+} transients (35–38). However, it has more recently been shown that constitutive NOS isoforms contribute to the heart failure phenotype. For example, NOS3 signaling may be enhanced in heart failure. This phenomenon can result from alterations in its regulatory pathways, [for example, β_3-AR signaling (39, 40) or alterations in caveolin (28)]. Damy *et al.* (34) demonstrated a disruption of the spatial localization of NOS1 (translocation from SR to sarcolemma) in tissue from patients with cardiomyopathy. Moreover, NOS1 was demonstrated to be up-regulated in these conditions. In the sarcolemma, NOS may inhibit contractility by modulating L-type Ca^{2+} channels. Because Arg is up-regulated in a number of pathophysiologic states, it is interesting to speculate whether Arg up-regulation may contribute to the pathogenesis of heart failure.

Arg, L-Arginine Pools, and Reciprocal Regulation of NOS. Although the concept of reciprocal regulation of NOS by Arg is not a novel concept in the biology of NO, its role in the heart has not been determined. The concept that Arg may regulate NOS activity and NO production originated with the description of the up-regulation

of Arg in macrophages. Endotoxin (lipopolysaccharide) administration in macrophages resulted in the coinduction of the Arg isoforms Arg I and Arg II, and inducible NOS (iNOS), leading to the hypothesis that Arg may limit sustained overproduction of NO by limiting substrate availability to iNOS (12, 26, 41, 42). Recently, Arg I and Arg II expression have been demonstrated in the rat lung, where they modulate cholinergic airway responses and NO activity (43). Arg I and Arg II expression has also been demonstrated in the penis (11, 16) and A293 cells overexpressing NOS1 (44), where reciprocal regulation of Arg and constitutive NOS1 exists. Our published data (10) and those of other researchers (20, 45, 46) support the notion that Arg isoforms are expressed constitutively in vascular endothelium and may (as in the airway, the penis, and A293 cells) modulate NOS activity by regulating L-arginine availability.

The intracellular concentration of L-arginine in endothelial cells exceeds its K_m for the NOS enzyme by 2- to 3-fold, indicating that L-arginine availability should not limit NOS activity or NO production. Also, exogenous L-arginine administration should not influence NOS activity and NO production. However, in certain conditions (diabetes, hypertension, and hypercholesterolemia), the addition of extracellular L-arginine enhances NO-dependent relaxation, giving rise to the "arginine paradox" and suggesting that factors other than L-arginine concentrations also influence L-arginine bioavailability. One such influence is the enzyme Arg, which we suggest competes with NOS for L-arginine. Also, spatial confinement of NOS1 and Arg suggests very tight control of L-arginine availability. Furthermore, the presence of endogenous NOS inhibitors may further exacerbate this paradox. Last, the presence of distinct intracellular L-arginine pools may be important in determining substrate availability.

Our data demonstrating that exogenous L-arginine had no effect on myocyte NO production or myocyte contractility is consistent with the idea of different L-arginine pools in cardiac myocyte specifically but in other cells in general. Although there is very little available information regarding the pools of L-arginine available for NOS1 in the cardiac myocyte (47, 48), we may translate some of the concepts from the limited literature in endothelial cells. The fact that exogenous L-arginine in our experiments has little effect on NOS activity in the myocyte suggests that the pool of L-arginine that is available to NOS^- may not be regulated by the CAT transporter. This notion is consistent with the data from Closs and coworkers (21, 22), who have demonstrated that, in endothelial cells, there are three pools of L-arginine. The first pool (pool I) is regulated by the CAT transporter and can be depleted by L-lysine and restored by exogenous L-arginine. In contrast, pool II is accessible to endothelial NOS but is not freely exchangeable with extracellular L-lysine (or L-arginine). In endothelial cells, there are two components of pool II. The first component, IIA, which can be depleted by neutral amino acids, results from recycling of citrulline. Pool IIB, however, results from protein breakdown and is not responsive to either cationic or neutral AAs. Because Arg (specifically, Arg II) in mitochondria is the source of this pool, it would be appropriate to speculate that it is this pool (unaffected by extracellular L-arginine, but regulated by Arg) that is modulating contractility.

Mitochondrial Arg and SR Coupling. Although myocyte subcellular fractionation and immunoblotting suggested that Arg II is predominantly found in the mitochondria, immunoelectron microscopy conclusively demonstrated that Arg II is confined almost exclusively to the mitochondria. This observation is in agreement with the findings of others who demonstrate Arg II confined to the mitochondria in other cell types (49, 50) and is consistent with the putative N-terminal mitochondrial-targeting presequence found in the gene for Arg II (25, 26). However, coimmunoprecipitation experiments and WB demonstrated that Arg II is also found in crude SR preparations and immunoprecipitates of NOS1 (known to be found in the SR). Also, SR proteins (SERCA) were demonstrated in mitochondrial isolates and mitochondrial proteins in crude SR fractions. This observation shows the tight spatial association and signal coupling between the mitochondria and machinery involved in excitation–contraction coupling (for example, the RYR channel). This interaction is critical because of the need for continuous regulation of the cellular oxidative energy generation in the mitochondria to the contractile work performed (for review, see ref. 51). Thus, our findings of Arg II expression in both mitochondria and SR fractions (most likely contaminated with mitochondrial membrane) are not inconsistent. Also, they support the idea that mitochondrial Arg II may regulate concentrations of L-arginine in the microdomain of NOS1, thus modulating RYR function. Although purely speculative, this interaction may represent a mechanism whereby metabolism is coupled to the fine-tuning of contractility. Also, it raises the question, with regard to spatial confinement of NOS1 signaling, of whether the SR and mitochondria can be regarded as one microdomain. Fig. 6 is a schematic representation of our proposed model of how Arg II may constrain NOS1 activity and, thus, contractility by regulating the SR/mitochondrial L-arginine microdomain concentration.

Conclusion

Arg is expressed in the heart and is located in myocyte mitochondria, where it regulates NO-dependent basal myocardial contractility in a NOS1-dependent manner. These findings contribute to our understanding of the importance of spatial confinement of NO signaling in the heart, the potential importance of substrate limitation in the regulation of NOS activity, and the potential role or Arg as a target for treatment of myocardial dysfunction in which NO signaling is disrupted.

Methods

Reagents. BEC and Nor-NOHA were obtained from Calbiochem. The rest of the chemical reagents were obtained from Sigma.

Animals. Mice (8–10 weeks old) that were homozygous for targeted disruption of the NOS1 gene ($NOS1^{-/-}$, $n = 3$), the NOS3 gene ($NOS3^{-/-}$, $n = 3$), and WT control mice (WT, C57BL/6J, $n = 3$) were purchased from The Jackson Laboratory. All rats (Wistar; 11–14 weeks old) were purchased from Harlan Laboratories (Haslett, MI). All protocols conformed to the National Institutes of Health and American Physiological Society Guidelines for the Use and Care of Laboratory Animals.

WB and Coimmunoprecipitation. Heart tissue and isolated cardiac myocyte protein of lysates were immunoprecipitated with or without 2 μg of NOS3 (BD Biosciences, Franklin Lakes, NJ) Arg II, or NOS1 (Santa Cruz Biotechnology) Abs overnight at 4°C. After

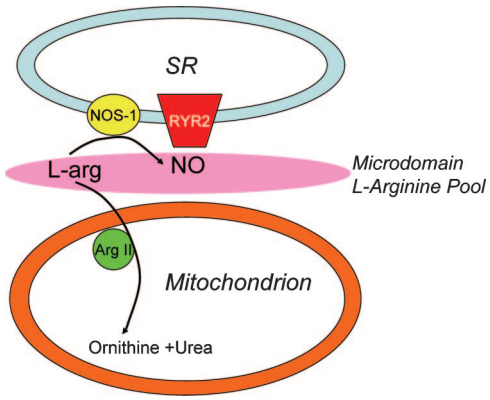

Fig. 6. Schematic representation of the proposed mechanism by which mitochondrial Arg II regulates NOS1-dependent myocardial contractility.

incubation with protein A/G agarose for 4 h at 4°C, the beads were washed with lysis buffer three times. Agarose beads were subjected to SDS/PAGE sample buffer, resolved on a 10% SDS/PAGE, and immunoblotted with a mAb against NOS1, NOS3 mAb, or Arg II polyclonal Ab (overnight at 4°C, 1:1,000 dilution). Ab was detected with enhanced chemiluminescence system (Amersham Pharmacia).

For RT-PCR, total RNA from rat heart and isolated myocytes was prepared by homogenization in the presence of Trizol reagent (GIBCO), and RT-PCR was performed with specific Arg I and II primers, as described (52).

Immunofluorescence. Isolated myocytes from rabbit were fixed with acetone/ethanol (3:7, vol/vol) solution at 4°C overnight and permeabilized with 3% paraformaldehyde and 0.5% Triton X-100 in PBS, rinsed with PBS, and incubated with mAb against Arg I (BD Biosciences) or polyclonal Ab against Arg II and then with DAPI-conjugated anti-mouse IgG or Cy5-conjugated-anti-rabbit IgG Ab. Washed myocytes were examined with a confocal fluorescence microscope (LSM 410; Zeiss).

Isolation of SR and Mitochondria Preparation. We prepared SR fractions according to the method described by Khan *et al.* (4). Purified SR fractions were resolved electrophoretically and probed with anti-Arg II, anti-SR Ca^{2+} ATPase (Affinity BioReagents, Golden, CO), and anti-NOS1 Abs.

Mitochondria were prepared by using the mitochondria-isolation kit for tissue (Pierce).

Immunoelectron Microscopy. Immunoelectron microscopy was performed by standard procedures. Briefly, adult Wistar rats were deeply anesthetized, and hearts were removed and retrogradely perfused with 4% paraformaldehyde–0.05% glutaraldehyde in PBS and postfixed overnight at 4°C. One-hundred-micrometer-thick vibratome sections were cut and collected in PBS, followed by incubation in the primary Abs (rabbit anti-Arg-II; 1:50 dilution) for 24 h at 4°C. After washing, the secondary Abs labeled with 6-nm gold particles were applied, and the tissue sections were examined with an electron microscope.

Arg Activity. Rat hearts and myocytes were homogenized in lysis buffer (50 mM Tris·HCl, pH 7.5/0.1 mM EDTA, with protease inhibitor) and centrifuged for 30 min at 14,000 × *g* at 4°C for an Arg-activity assay, as described (20).

NOS Activity and NO Production. NO production was evaluated by measuring nitrite levels (Calbiochem) after preincubation of heart and myocytes with BEC (10 μmol/liter) in PBS (pH 7.4), as described (52).

Measurement of Contractility in Isolated Rat and Mouse Myocytes. Both rat and mouse myocytes were isolated by enzymatic digestion as described (2, 3). Myocytes were transferred to a lucite chamber on the stage of an inverted microscope (TE 200; Nikon), continuously superfused with Tyrode's solution containing 1.0 mM Ca^{2+}, and stimulated at 1 Hz. Sarcomere length was recorded with an IonOptix (Milton, MA) intensified charged-coupled device camera. Change in average sarcomere length was determined by fast Fourier transform of the *Z*-line density trace to the frequency domain as described (2, 3).

Data Analysis and Statistics. All data are presented as mean ± SEM, with *N* indicated for each experimental protocol. For dose-responses, data were fitted by using the software program PRISM 4 (Graphpad, San Diego), and E_{max} and EC_{50} were calculated. Statistical analysis was performed by using one-way ANOVA with posttest or unpaired Student's *t* test, as appropriate.

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