Expression of Inducible Nitric Oxide Synthase Depresses β-Adrenergic–Stimulated Calcium Release From the Sarcoplasmic Reticulum in Intact Ventricular Myocytes

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Background—β-Adrenergic hyporesponsiveness in many cardiomyopathies is linked to expression of inducible nitric oxide synthase (iNOS) and increased production of NO. The purpose of this study was to examine whether iNOS expression alters the function of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channel (ryanodine receptor, RyR) during β-adrenergic stimulation.

Methods and Results—Expression of iNOS was induced by lipopolysaccharide (LPS) injection (10 mg/kg) 6 hours before rat myocyte isolation. Confocal microscopy (fluo-3) was used to measure Ca\(^{2+}\) spark frequency (CaSpF, reflecting resting RyR openings) and Ca\(^{2+}\) transients. CaSpF was greatly increased by the adenylate cyclase activator forskolin (100 nmol/L) in normal myocytes (iNOS not expressed), but this effect was suppressed (by 77%) in LPS myocytes (iNOS expressed). When NO production by iNOS was inhibited by aminoguanidine (1 mmol/L), there was a further increase in the forskolin-induced CaSpF in LPS myocytes (to levels similar to the forskolin-stimulated CaSpF in normal myocytes). This effect was also seen in myocytes isolated from a failing human heart. There was no effect of aminoguanidine on forskolin-stimulated CaSpF in normal myocytes. ODQ (10 \(\mu\)mol/L), an inhibitor of NO stimulation of guanylate cyclase, did not restore the forskolin-induced rise in CaSpF in LPS myocytes. Aminoguanidine also increased twitch Ca\(^{2+}\) transient amplitude in LPS myocytes after forskolin application (independent of changes in SR Ca\(^{2+}\) load).

Conclusions—iNOS/NO depresses β-adrenergic–stimulated RyR function through a cGMP-independent pathway (eg, NO- and/or peroxynitrite-dependent redox modification). This mechanism limits β-adrenergic responsiveness and may be an important signaling pathway in cardiomyopathies, including human heart failure. (Circulation. 2001;104:2961-2966.)

Key Words: receptors, adrenergic, beta • nitric oxide • sarcoplasmic reticulum • calcium • heart failure

Calcium regulates cardiac contraction via excitation-contraction (EC) coupling. Diseased human hearts exhibit deterioration of contractile function, frequently seen as decreased β-adrenergic responsiveness. β-Adrenergic activation stimulates adenylate cyclase, increases cAMP, and activates cAMP-dependent protein kinase (PKA). Many key EC coupling proteins are phosphorylated by PKA; for example, the sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channels (ryanodine receptors, RyR). The RyR is responsible for Ca\(^{2+}\) sparks. Ca\(^{2+}\) sparks can occur stochastically during diastole (independent of Ca\(^{2+}\) influx), but the cellular Ca\(^{2+}\) transient is due to temporal and spatial summation of Ca\(^{2+}\) sparks synchronized by the L-type calcium channel.

Although there is downregulation of β-adrenergic receptors in end-stage heart failure, many cardiomyopathies also increase cytokine production, which leads to the expression of inducible nitric oxide synthase (iNOS) in cardiac myocytes (eg, heart failure). NO is a signaling molecule that is important in regulating myocardial contractility. Ventricular myocytes possess 2 NOS isoforms, constitutive (cNOS, type III) and inducible (iNOS, type II). iNOS expression leads to continuous production of large amounts of NO, compared with the small amount of NO produced by cNOS. Once NO is produced, it has 2 signaling pathways, cGMP-dependent and cGMP-independent, and can contribute to cardiac dysfunction. In fact, many studies have found that when cardiac myocytes express iNOS, contractile function is decreased. The mechanism of iNOS-dependent contractile dysfunction in human and animal cardiomyopathies, however, is not yet clear. It has been shown that at least part of the contractile dysfunction associated with these various diseases is due to overproduction of NO caused by expression of iNOS. NO has been shown to have “antiadrenergic” effects on cardiac myocyte function. Depending on experimental con-
ditions and/or species, NO typically does not affect basal function but causes a hyporesponsiveness to β-adrenergic stimulation.\textsuperscript{11,12,15} NO also affects RyR activity in lipid bilayers\textsuperscript{13} and thus could contribute to the antiadrenergic effect of NO. Little work has been done, however, on the effects of NO on SR function, especially during β-adrenergic stimulation, in intact cardiac myocytes that express iNOS.

The objective of this study is to examine the effect of iNOS expression on Ca\textsuperscript{2+} sparks, Ca\textsuperscript{2+} transients, and RyR activity during β-adrenergic stimulation. The results may have direct bearing on the β-adrenergic hyporesponsiveness commonly seen in cardiomyopathies. We hypothesize that iNOS expression and consequent NO production decrease Ca\textsuperscript{2+} transients because of inhibition of RyR activity during β-adrenergic stimulation.

Methods

Expression of iNOS and Rat Cardiac Myocyte Isolation

Expression of iNOS was induced by injection of lipopolysaccharide (LPS, 10 mg/kg IP) into adult Sprague-Dawley rats (250 to 300 g) as described by Luss et al.\textsuperscript{17} Ventricular myocytes were isolated 6 hours after LPS injection as previously described.\textsuperscript{13} All procedures and animal care were in accordance with American Association for Accreditation of Laboratory Animal Care guidelines.

Human Myocyte Isolation

Myocytes were isolated from a failing human heart (ischemic, 63-year-old man) obtained at the time of transplantation. A section of left ventricle was excised. A vessel was cannulated and perfused with cold Tyrode’s solution containing (in mmol/L) NaCl 120, KCl 20, MgCl\textsubscript{2} 1, glucose 10, HEPES 10, NaH\textsubscript{2}PO\textsubscript{4} 0.33, taurine 20, and pyruvate 5, and 3 mmol/L 2,3-butanedione monoxime (BDM, inhibitor of contractile proteins) to arrest the heart. Tissue was then perfused with the above solution (37°C) (except 4 mmol/L KCl) for 10 minutes, then collagenase (0.8 mg/mL, Worthington type II) and protease (0.08 mg/mL, Sigma) were added for 40 minutes, followed by washout with Tyrode’s solution for 10 minutes. Then the tissue was taken down, minced, and triturated. Cells were allowed to gravity-settle twice for 10 minutes, and the supernatant was discarded. The cell pellet was resuspended in Tyrode’s solution with 200 μmol/L Ca\textsuperscript{2+} without BDM. Cells were used within 6 hours of isolation. The procedure was done according to Loyola University Medical Center Institutional Review Board–approved protocol.

Dye Loading and Fluorescence Imaging

Myocytes were loaded with fluo-3-AM, and fluorescence imaging was performed as previously described.\textsuperscript{4} Ca\textsuperscript{2+} transients were derived from changes in fluorescence intensities (F) and normalized to basal fluorescence (F\textsubscript{0}) and expressed as F/F\textsubscript{0}.

Experimental Protocols

Myocytes were field-stimulated via platinum electrodes (0.2 Hz) and superfused with normal Tyrode’s solution (room temperature). When Ca\textsuperscript{2+} transients reached steady state, stimulation was stopped, and resting Ca\textsuperscript{2+} sparks were measured for 13.4 seconds. Field stimulation was started again and the solution switched to normal Tyrode’s solution with forskolin (100 nmol/L). When the cell response reached steady state (4 minutes), resting Ca\textsuperscript{2+} sparks were measured. Field stimulation was started again and the solution switched to normal Tyrode’s solution with forskolin and aminoguanidine (1 mmol/L) until steady state (4 minutes), and resting Ca\textsuperscript{2+} sparks were measured. SR Ca\textsuperscript{2+} content was estimated by rapid application of 10 mmol/L caffeine dissolved in normal Tyrode’s solution.

Results

iNOS Expression and Ca\textsuperscript{2+} Spark Frequency After β-Adrenergic Stimulation

We examined the effect of iNOS expression in cardiac myocytes and its effect on resting Ca\textsuperscript{2+} spark frequency (CaSpF) after β-adrenergic stimulation. Figure 1 shows representative experiments performed on a single myocyte isolated from normal (control) and LPS rats. Shown are line-scan images obtained with confocal microscopy. During control superfusion, there were few Ca\textsuperscript{2+} sparks (Figure 1, top) in either normal or LPS myocytes. When the cell was superfused with forskolin (100 nmol/L), a direct activator of adenylate cyclase, there was a substantial increase in the CaSpF in normal myocytes but only a small increase in the LPS myocytes (Figure 1, middle). When the cell was superfused with forskolin plus aminoguanidine (1 mmol/L), a specific iNOS inhibitor, there was no further increase in CaSpF in the normal myocyte but a dramatic increase in CaSpF in the LPS myocyte (Figure 1, bottom). Pooled data (Figure 2) show that there was no statistical difference between normal and LPS myocyte basal CaSpF [15 ± 7 versus 8 ± 2 sparks/(pL·s), P = NS]. There was a significant attenuation, however, in the response to forskolin in LPS myocytes compared with the normal myocytes [29 ± 9 versus 128 ± 14 sparks/(pL·s), P < 0.05]. Blocking iNOS with aminoguanidine had no further effect on forskolin-stimulated CaSpF in normal myocytes [131 ± 21 sparks/(pL·s)] but significantly increased the LPS myocyte response to forskolin stimulation [to 115 ± 30 sparks/(pL·s), P < 0.05 compared with forskolin alone]. In fact, there was no statistical difference between normal and LPS myocyte CaSpF in the presence of forskolin + aminoguanidine, suggesting complete reversal of the dysfunction. Moreover, these results may indicate that NO (produced via iNOS) can limit the β-adrenergic stimulation of CaSpF.

Guanylate Cyclase Involvement in the iNOS-Induced Depression of Ca\textsuperscript{2+} Sparks

If the CaSpF limitation by iNOS expression is due to NO activation of guanylate cyclase and increased cGMP production, then inhibiting guanylate cyclase should ameliorate the reduced CaSpF (forskolin-stimulated). Figure 3 shows that ODQ (10 μmol/L), a specific inhibitor of NO stimulation of guanylate cyclase, had no effect on the forskolin-induced increase in CaSpF in myocytes isolated from LPS rats. This contrasts to when iNOS was inhibited with aminoguanidine.
These data suggest that the effect of NO on CaSpF is independent of guanylate cyclase activation and cGMP but could be mediated by other actions (eg, nitrosylation or other NO- or peroxynitrite-dependent redox modification of RyR).

Effects of iNOS on Twitch Ca$^{2+}$ Transients and SR Ca$^{2+}$ Load After β-Adrenergic Stimulation

We examined the effects of iNOS expression on twitch [Ca$\text{i}$] and SR Ca$^{2+}$ load in cardiac myocytes field-stimulated at 0.2 Hz. SR Ca$^{2+}$ load was measured by rapid application of caffeine (10 mmol/L). Figure 4A shows typical Ca$^{2+}$ transient recordings and SR Ca$^{2+}$ load of a myocyte isolated from an LPS rat. Figure 4B shows the mean data of twitch [Ca$\text{i}$]. Forskolin (100 nmol/L) doubled twitch Δ[Ca$\text{i}$], compared with control (5.4±0.4 versus 2.6±0.7 F/F$\text{o}$, P<0.05). When the cell was superfused with aminoguanidine, however, there was an additional increase in twitch Δ[Ca$\text{i}$] (7.0±0.7 F/F$\text{o}$, P<0.05 versus forskolin alone). A main determinant of SR Ca$^{2+}$ release is SR Ca$^{2+}$ load. We examined this by caffeine-induced Δ[Ca$\text{i}$] (Figure 4A and 4C). Figure 4C shows mean data of SR Ca$^{2+}$ load. As expected, forskolin increased SR Ca$^{2+}$ load compared with control in LPS myocytes (6.0±0.8 versus 5.7±0.6 F/F$\text{o}$, P<0.05). Superfusion with aminoguanidine had no further effect on SR Ca$^{2+}$ load (8.6±0.6 F/F$\text{o}$, P=NS versus forskolin alone). We conclude that the aminoguanidine-induced increase in twitch Δ[Ca$\text{i}$] (and CaSpF in Figures 1 and 2) in the presence of forskolin is independent of changes in SR Ca$^{2+}$ load.

iNOS Inhibition and CaSpF in Human Failing Myocytes

We investigated whether iNOS inhibition would enhance the β-adrenergic response of myocytes isolated from a failing human heart. The same experimental protocol was followed as with the LPS rat. Figure 5A shows line-scan images obtained with confocal microscopy. During control superfusion, there were few Ca$^{2+}$ sparks (top). Addition of isoproterenol (10 nmol/L), a β-adrenergic agonist, caused a small increase in CaSpF (middle). When 1 mmol/L aminoguanidine was added, there was a further increase in the CaSpF (bottom). Figure 5B shows the mean±SEM of all sweeps.

Discussion

Many studies have found that iNOS expression and increased NO production decrease contractile function. This may be a...
major pathway in the diminished β-adrenergic responsiveness seen in many cardiomyopathies. Little work has been done, however, to assess the cellular mechanism of iNOS/NO-induced dysfunction.

β-Adrenergic stimulation leads to a positive inotropic effect through activation of PKA and phosphorylation of several proteins involved in EC coupling. SR Ca\(^{2+}\) release events mediated by RyR have been identified as Ca\(^{2+}\) sparks, and these are the elementary events underlying EC coupling. 16

iNOS, NO Production, Myocardial Dysfunction, and LPS

The LPS model has been well characterized in cardiac myocytes. 14 For example, it is known that exposure to LPS leads to iNOS expression in cardiac myocytes, 14,17–20 increased NO synthase activity, 14,17–19 and increased cardiac myocyte cGMP levels. 20

Exposure to LPS leads to depressed myocardial contractility (usually seen as a decreased response to β-adrenergic stimulation) and has been shown to be mainly through induction of iNOS and the resultant increase in NO production. For example, a study using iNOS-knockout mice found that nearly all of the myocardial dysfunction due to LPS injection was attributable to iNOS expression. 21 Other studies have also found that inhibition of NO production reversed the contractile dysfunction due to LPS injection. 17,20 Thus, it is well established that LPS injection leads to cardiac myocyte iNOS functional expression and increased NO production and that this causes myocardial dysfunction.

NO, β-Adrenergic Stimulation, and Ca\(^{2+}\) Sparks

β-Adrenergic stimulation increases resting CaSpF. 15 This effect is most likely via increased SR Ca\(^{2+}\) load (secondary to phospholamban phosphorylation) and/or phosphorylation of RyR by PKA to increase \(P_{o}\). To the best of our knowledge, this is the first study to show that iNOS expression limits the increase in CaSpF induced by β-adrenergic stimulation. In intact ventricular myocytes isolated from the LPS rat model, which expresses iNOS, 14 there was a 77% decrease of the β-adrenergic–induced stimulation of CaSpF compared with control myocytes (not expressing iNOS). Specific inhibition of iNOS with aminoguanidine had no effect on normal myocytes (CaSpF was 102 ± 8% of forskolin control) but fully restored (compared with normal myocytes) the increase in CaSpF induced by forskolin in the myocytes from LPS rats (Figures 1 and 2). These data indicate that we can completely reverse this dysfunction simply by short-term (≤5 minutes) inhibition of iNOS. We have previously shown that aminoguanidine is a specific inhibitor of iNOS in cardiac myo-

![Figure 4. Effects of forskolin (FORSK) and aminoguanidine (AG) on twitch and caffeine-induced Ca\(^{2+}\) transients. A, Typical recordings of twitch [Ca], transients (expressed as F/F0) and transients induced by rapid caffeine application (to measure SR Ca\(^{2+}\) load). B, Pooled data for twitch ∆[Ca], transients (mean±SEM, ANOVA, *\(P<0.05\) vs control [CONT]; **\(P<0.05\) vs CONT and FORSK, \(n=8\) cells). C, Pooled data for SR Ca\(^{2+}\) load (mean±SEM, ANOVA, *\(P<0.05\) vs control, \(n=8\) cells).](http://circ.ahajournals.org/)

![Figure 5. Ca\(^{2+}\) sparks in failing human ventricular myocytes. Recordings of line-scan images of Ca\(^{2+}\) sparks (A). Line plots of [Ca] taken from selected sites (white bars) are shown under images. Top, Resting Ca\(^{2+}\) sparks in presence of control Tyrode’s solution (CONT), 1 spark shown in image. Middle, In presence of 10 nmol/L isoproterenol (ISO), 1 spark shown in image. Bottom, With ISO and 1 mmol/L aminoguanidine (ISO+AG), 5 sparks shown in image. B, Pooled data of CaSpF (mean±SEM, \(n=48\) sweeps from 2 cells).](http://circ.ahajournals.org/)
cytes. Thus, decreasing RyR activity may be a key mechanism in NO-induced reduction of β-adrenergic responsiveness.

cGMP-Independent or -Dependent Signaling?
NO has 2 signaling pathways: cGMP-dependent and cGMP-independent. Our results with ODQ, a specific inhibitor of NO stimulation of guanylate cyclase, imply that the effect of NO on CaSpF is independent of guanylate cyclase and cGMP (Figure 3). Previous work has shown that ODQ is a specific inhibitor of guanylate cyclase in cardiac myocytes (ODQ selectively decreased cGMP levels in response to NO donors). If NO inhibits CaSpF through a cGMP-dependent pathway, then ODQ should have further increased the forskolin response (as for aminoguanidine). Because ODQ did not have any effect, these data suggest that NO alteration of RyR activity is cGMP-independent. Other studies have shown that NO can alter RyR gating in lipid bilayers via nitrosylation. It is also known that the iNOS expression and increased NO production leads to peroxynitrite formation, which also causes myocardial dysfunction. We have shown that part of the antiadrenergic effect of NO is via NOx (eg, peroxynitrite). The NO pathway could also play a role in the depressed RyR activity seen in this LPS model. This present study does not delineate the cGMP-independent pathway of iNOS-induced reduction in RyR activity, but it could be via RyR nitrosylation, nitration, or some other NO-dependent pathway.

iNOS, Ca2+ Transients, and SR Ca2+ Load
In the present study, we found that iNOS expression limited the increase in twitch Δ[Ca2+]i, induced by forskolin (Figure 4A and 4B), although this effect was not as profound as was seen for CaSpF (Figure 2). A main determinant of SR Ca2+ release is the Ca2+ load of the SR. Figure 4C shows that forskolin caused an increase in SR Ca2+ load. Aminoguanidine, however, did not cause a further increase in SR Ca2+ load (Figure 4C). This implies that the decrease in Δ[Ca2+]i (Figure 4B, FORSK versus FORSK + AG) and CaSpF (Figure 2) with iNOS is not due to decreased SR Ca2+ load but may be a direct effect of NO (or peroxynitrite) on RyR gating during EC coupling. In addition, we showed that although NO donors do not alter basal CaSpF in normal myocytes, they do have prominent effects after β-adrenergic stimulation (mediated by altered SR Ca2+ load). This differs from results in the present study. We speculate that iNOS might be localized near RyR, thereby causing more direct local effects on RyR. With exogenous NO donors, local [NO] may not be high enough to directly affect RyR (the short half-life of NO may also limit local [NO] in this context). Neuronal NOS (NOS I) might also be localized in the SR, but this is unlikely to be relevant here, because it would not be aminoguanidine-sensitive.

NO and Human Heart Failure
It is known that NO (produced via NO donors and endotoxin-induced iNOS expression) depresses the β-adrenergic response in human myocardium. iNOS expression has been reported in cardiac myocytes from various human heart failure phenotypes. In fact, there is a significant corre-
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