

Research Article

Contractile Activity Regulates Inducible Nitric Oxide Synthase Expression and NO_i Production in Cardiomyocytes via a FAK-Dependent Signaling Pathway

Mienscheng Chu,¹ Yevgeniya Koshman,¹ Rekha Iyengar,¹ Taehoon Kim,¹
Brenda Russell,² and Allen M. Samarel¹

¹ Cardiovascular Institute, Loyola University Chicago Stritch School of Medicine, 2160 South First Avenue, Maywood, IL 60153, USA

² Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL 60612, USA

Correspondence should be addressed to Allen M. Samarel, asamare@lumc.edu

Received 29 March 2012; Revised 6 June 2012; Accepted 6 June 2012

Academic Editor: J. Adolfo García-Sáinz

Copyright © 2012 Mienscheng Chu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Intracellular nitric oxide (NO_i) is a physiological regulator of excitation-contraction coupling, but is also involved in the development of cardiac dysfunction during hypertrophy and heart failure. To determine whether contractile activity regulates nitric oxide synthase (NOS) expression, spontaneously contracting, neonatal rat ventricular myocytes (NRVM) were treated with L-type calcium channel blockers (nifedipine and verapamil) or myosin II ATPase inhibitors (butanedione monoxime (BDM) and blebbistatin) to produce contractile arrest. Both types of inhibitors significantly reduced iNOS but not eNOS expression, and also reduced NO_i production. Inhibiting contractile activity also reduced focal adhesion kinase (FAK) and AKT phosphorylation. Contraction-induced iNOS expression required FAK and phosphatidylinositol 3-kinase (PI(3)K), as both PF573228 and LY294002 (10 μM, 24 h) eliminated contraction-induced iNOS expression. Similarly, shRNAs specific for FAK (shFAK) caused FAK knockdown, reduced AKT phosphorylation at T308 and S473, and reduced iNOS expression. In contrast, shRNA-mediated knockdown of PYK2, the other member of the FAK-family of protein tyrosine kinases, had much less of an effect. Conversely, overexpression of a constitutively active form of FAK (CD2-FAK) or AKT (Myr-AKT) reversed the inhibitory effect of BDM on iNOS expression and NO_i production. Thus, contraction-induced iNOS expression and NO_i production in NRVM are mediated via a FAK-PI(3)K-AKT signaling pathway.

1. Introduction

Intracellular nitric oxide (NO_i) is a free radical that is synthesized by a family of nitric oxide synthases (NOSs), consisting of endothelial NOS (eNOS or NOS1), inducible NOS (iNOS or NOS2), and neuronal NOS (nNOS or NOS3) isoforms. All three isoforms are expressed in cardiomyocytes. eNOS and nNOS are constitutively expressed, whereas iNOS is up-regulated under pathological conditions such as sepsis, hypertension, hypertrophy and heart failure [1, 2]. Thus, NO_i production plays an important role in regulating cardiomyocyte function during excitation-contraction coupling, mitochondrial respiration, hypertrophic remodeling, apoptosis, and myocardial regeneration.

The local effects of endogenous NO_i production on cardiomyocyte structure and function are dependent upon the localization of the different NOS isoforms. For instance, eNOS is associated with caveolin-3 within caveoli, where it locally regulates β-adrenergic receptor stimulation [3]. nNOS is associated with sarcoplasmic reticulum (SR) membranes, where it modulates SR Ca²⁺ uptake and release [2–4]. During different pathological conditions, NO_i production shifts from spatially and temporally regulated NO_i production to dysregulated, excessive release. The relative abundance of NOS expression and activity in diseased hearts also changes, with nNOS and iNOS being upregulated, whereas eNOS is downregulated [5]. During these pathological states, iNOS is localized throughout the cytoplasm and increased

iNOS expression and NO_i production then contribute to myocardial dysfunction and reduced myocardial responsiveness to β -adrenergic stimulation [6–8]. Previous studies have demonstrated that iNOS expression is significantly increased in the intact, volume-overloaded heart [7], which is mediated in part by systemic production of TNF- α , IL-1 β , angiotensin II, and other cytokines [1, 9]. However, iNOS expression and NO_i production can also be induced *ex vivo* by static stretch of cultured neonatal rat ventricular myocytes (NRVM) [10], suggesting the involvement of a mechanosensitive signaling pathway in cardiomyocytes that operates independently of circulating growth factors and cytokines. Indeed, cardiomyocytes can directly transduce physical forces into biochemical signals and generate appropriate responses leading to alterations in cellular structure and function [11]. Costameres (and focal adhesions, their functional equivalent in cultured cells) are important subcellular structures responsible for mechanotransduction in cardiomyocytes (for review, see [12]). A complex signaling web connects mechanosensory and growth factor-dependent signal transduction pathways in cardiomyocytes, and many different downstream effectors are activated in response to mechanical loading. Mechanotransduction via integrins and their accessory nonreceptor protein tyrosine kinases (focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (PYK2)) resembles the downstream signals generated following activation of receptor tyrosine kinases, and their interconnecting signaling pathways share critical components with signaling pathways activated in response to peptide growth factors and cytokines [13]. These responses include acute alterations in contractile function, as well as long-term, structural changes in cardiomyocyte size, shape, and gene expression.

In a previous study, we demonstrated that electrical pacing of quiescent, freshly isolated adult cat ventricular myocytes acutely increased NO_i production by a mechanochemical signaling pathway that required Ca²⁺-calmodulin (CaM), phosphatidylinositol-3-kinase (PI(3)K), and AKT [14]. Based upon relatively specific small-molecule inhibitors of the various NOS isoforms, acute stimulation of NO_i production by electrical pacing and mechanical activity involved both eNOS and nNOS. In this paper, we examined whether cardiomyocyte contractile activity also regulated NO_i production over a longer time period, via the contraction-dependent upregulation of iNOS expression in spontaneously contracting NRVM. We also investigated the roles of FAK, PYK2, PI(3)K, and AKT in this process.

2. Methods

2.1. Reagents. PC-1 tissue culture medium was obtained from BioWhittaker (Walkersville, MD, USA). Dulbecco's Modified Eagle Medium (DMEM) and Medium 199 were obtained from Gibco BRL (Grand Island, NY, USA). FAK, iNOS, eNOS and C-terminal PYK2/CRNK monoclonal antibodies (mAb) were purchased from BD Transduction Laboratories (San Jose, CA, USA). Phospho-specific FAK-Y397 and FAK-Y577 polyclonal (pAb) antibodies were

purchased from BioSource International, Camarillo, CA. Phospho-specific PYK2-Y402, AKT-T308, AKT-S473, and total AKT pAb were purchased from Cell Signaling Technology (Danvers, MA, USA). N-terminal PYK2 pAb was obtained from BioLegend (San Diego, CA, USA). GAPDH mAb was obtained from Novus Biologicals, Littleton, CO, USA. Horseradish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgGs were obtained from BioRad (Hercules, CA, USA). PF573228 and blebbistatin were obtained from Tocris Bioscience (Minneapolis, MN, USA). All other reagents were of the highest grade commercially available and were obtained from Sigma (St. Louis, MO, USA) and Baxter S/P (McGaw Park, IL).

2.2. Cell Culture. Animals used in these experiments were handled in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Council of the American Physiological Society. NRVMs were isolated from the hearts of 2-day old Sprague-Dawley rats by collagenase digestion, as previously described [15]. Cells were preplated for 1 h in serum-free PC-1 medium to reduce nonmyocyte contamination. The nonadherent NRVMs were then plated at a density of 1600 cells per mm² onto collagen-coated 60 mm dishes and left undisturbed in a 5% CO₂ incubator at 37°C for 36 h. Unattached cells were removed by aspiration, washed twice in HBSS, and the attached cells were maintained in a solution of DMEM/Medium 199 (4:1) containing antibiotic/antimycotic solution. At this density, spontaneous contractile activity (~100–150 beats per min) was visible within 24 h of plating. NRVMs were then infected (24 h) with replication-defective adenoviruses (Adv) diluted in DMEM/Medium 199. Medium was replaced with virus-free DMEM/Medium 199, treated with inhibitors or cultured for an additional 6–72 h.

2.3. Adenoviral Constructs. Advs expressing shRNAs specific for rat FAK (shFAK), rat PYK2 (shPYK2), and firefly luciferase (shLuc) were generated as previously described [16]. To inhibit FAK-dependent signal transduction, NRVMs were infected (5 moi, 24 h) with replication-defective adenoviruses (Adv) expressing either GFP-FRNK [17] or Y397F-FAK [18], the latter of which was kindly provided by Dr. T. Kasahara, Kyoritsu College of Pharmacy, Tokyo, Japan. To inhibit PYK2-dependent signaling, a replication-defective Adv-expressing FLAG-tagged, human Cell Adhesion Kinase- β Related Non-Kinase (CRNK) was kindly provided by Dr. Andrey Sorokin, Medical College of Wisconsin [19]. FAK-dependent signaling was increased using replication-defective Adv expressing either wildtype (WT) FAK [20] or a "constitutively active" FAK fusion protein (CD2-FAK) [21] which was constructed as previously described [16]. AKT-dependent signaling was increased using a replication-defective Adv-expressing either WT-AKT, or constitutively active AKT (Myr-AKT), which were kindly provided by Dr. K. Walsh, Tufts University School of Medicine, Boston, MA, USA [22]. In each experiment, a replication-defective Adv expressing either GFP [17] or nuclear-encoded β -galactosidase (ne β gal) [23] was used to control for nonspecific effects

of Adv infection. All Advs were propagated in HEK293 cells and purified by CsCl gradient centrifugation. The multiplicity of viral infection (moi) was determined by viral dilution assay in HEK293 cells grown in 96 well clusters. At moi of 5–10, >95% of the cells were infected, as determined by Adv-nef β gal infection and X-gal staining, and there were no cytotoxic effects of Adv infection during the 24–72 h following Adv infection.

2.4. SDS-PAGE and Western Blotting. NRVMs were homogenized in lysis buffer [24], and equal amounts of extracted proteins (50–100 μ g) were separated by SDS-PAGE and Western blotting on 10% polyacrylamide gels. Primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies and visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA). Developed films were then scanned on a HP Deskjet 4890 Scanner, and band intensity was quantified using UN-SCAN-IT Gel Software, Ver. 6.1 (Silk Scientific, Orem, UT). In all experiments, band intensity obtained with the phosphospecific antibody was divided by the band intensity for total protein (phosphorylated + nonphosphorylated) and then normalized to the intensity of the control sample.

2.5. NO_i Measurements. Measurements of NO_i production were obtained using the fluorescent NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2 DA) [25, 26] as previously described [27]. Cells were incubated with membrane-permeant DAF-2 DA (2 μ M; 40 min) at room temperature in 3 mL standard Tyrode solution containing 100 μ M L-arginine. Cells were subsequently washed for 30 min. DAF-2 fluorescence was excited at 480 nm and emitted cellular fluorescence was recorded at 540 nm. Cellular DAF-2 fluorescence intensity (F) was quantified with Image J Software. Control measurements of NO_i obtained in different experiments were grouped together and compared to drug treatment or adenoviral infection groups.

2.6. Data Analysis. Results were expressed as means \pm SEM. Normality was assessed using the Kolmogorov-Smirnov test. Data were compared using one-way ANOVA followed by Student-Newman-Keuls test, one-way ANOVA on Ranks followed by Dunn's test, Student-Newman-Keuls test, or paired t-test, where appropriate. Differences among means were considered significant at $P < 0.05$. Data were analyzed using SigmaPlot for Windows, Ver. 9.0 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Contractile Activity is Essential for iNOS Expression in NRVM. Spontaneously contracting NRVMs were treated with the L-type calcium channel blockers nifedipine and verapamil (which inhibit both spontaneous [Ca²⁺]_i transients and contractile activity) or the myosin II ATPase inhibitors butanedione monoxime (BDM) and blebbistatin (which block contractile activity but have relatively little

effects on spontaneous [Ca²⁺]_i transients) [14, 28–30]. iNOS and eNOS expression was then examined by SDS-PAGE and Western blotting under basal conditions and in response to contractile arrest. As seen in Figure 1(a), NRVM expressed substantial quantities of both eNOS and iNOS under basal conditions. Treatment with the various inhibitors of spontaneous contractile activity all significantly reduced iNOS expression in a dose-dependent fashion (Figures 1(b) and 1(c)), with little or no effect on eNOS expression levels (Figure 1(a)). As both types of contractile inhibitors substantially reduced iNOS expression, these results suggested that mechanical activity per se, rather than a Ca²⁺-dependent, contraction-independent signaling pathway was required. Next, we measured NO_i production using DAF-2-DA. As shown in Figure 1(d), nifedipine, BDM and blebbistatin all reduced NO_i production as compared to spontaneously contracting, control NRVM. Interestingly, nifedipine reduced NO_i production to a greater extent than either BDM or blebbistatin, suggesting that at the time of NO_i measurement, both [Ca²⁺]_i and mechanical activity contributed to enhanced NO_i production. The partial [Ca²⁺]_i dependence of NO_i production is consistent with the known role of Ca²⁺-CaM in regulating eNOS activity [31].

3.2. Contraction-Dependent iNOS Expression Requires FAK and PI(3)K. To investigate the role of FAK and PI(3)K in regulating contraction-dependent iNOS expression, NRVMs were first treated with PF573228 or LY294002, which are potent and highly selective inhibitors of FAK [32] and PI(3)K [33] activities, respectively. As seen in Figures 2(a) and 2(b), both agents substantially reduced iNOS levels in NRVM. Of note, at the concentrations used here, neither agent appeared to affect spontaneous contractile activity in these high-density cultures. To confirm the role of FAK in regulating iNOS expression, NRVMs were also infected with Adv-expressing shRNAs specific for FAK (shFAK) [16]. An Adv-expressing a shRNA specific for firefly luciferase (shLuc) was used to control for nonspecific effects of Adv infection and shRNA expression. As seen in Figure 2(c), shFAK but not shLuc substantially reduced FAK expression and also reduced iNOS expression. iNOS expression was also reduced by using 2 other inhibitors of FAK-dependent signaling (i.e., GFP-FRNK and Y397F-FAK; data not shown).

Previous studies have demonstrated that FAK autophosphorylation at Y397 provides a docking site for the binding and tyrosine phosphorylation of the p85 subunit of PI(3)K, which is required for its activation in NRVM [34, 35] and other cell types [36–41]. PI(3)K activation in turn leads to the activation of AKT. As both FAK and PI(3)K activities appeared necessary to regulate contraction-dependent iNOS expression, we next examined whether FAK was also involved in regulating AKT. As seen in Figures 3(a) and 3(b), spontaneously contracting NRVM demonstrated high levels of basal AKT activation, as detected by Western blotting with antibodies specific for AKT phosphorylated at T308 and S473. In contrast, NRVMs arrested with L-type Ca²⁺ channel blockers or inhibitors of myosin ATPase demonstrated reduced FAK and AKT phosphorylation. shRNA-induced

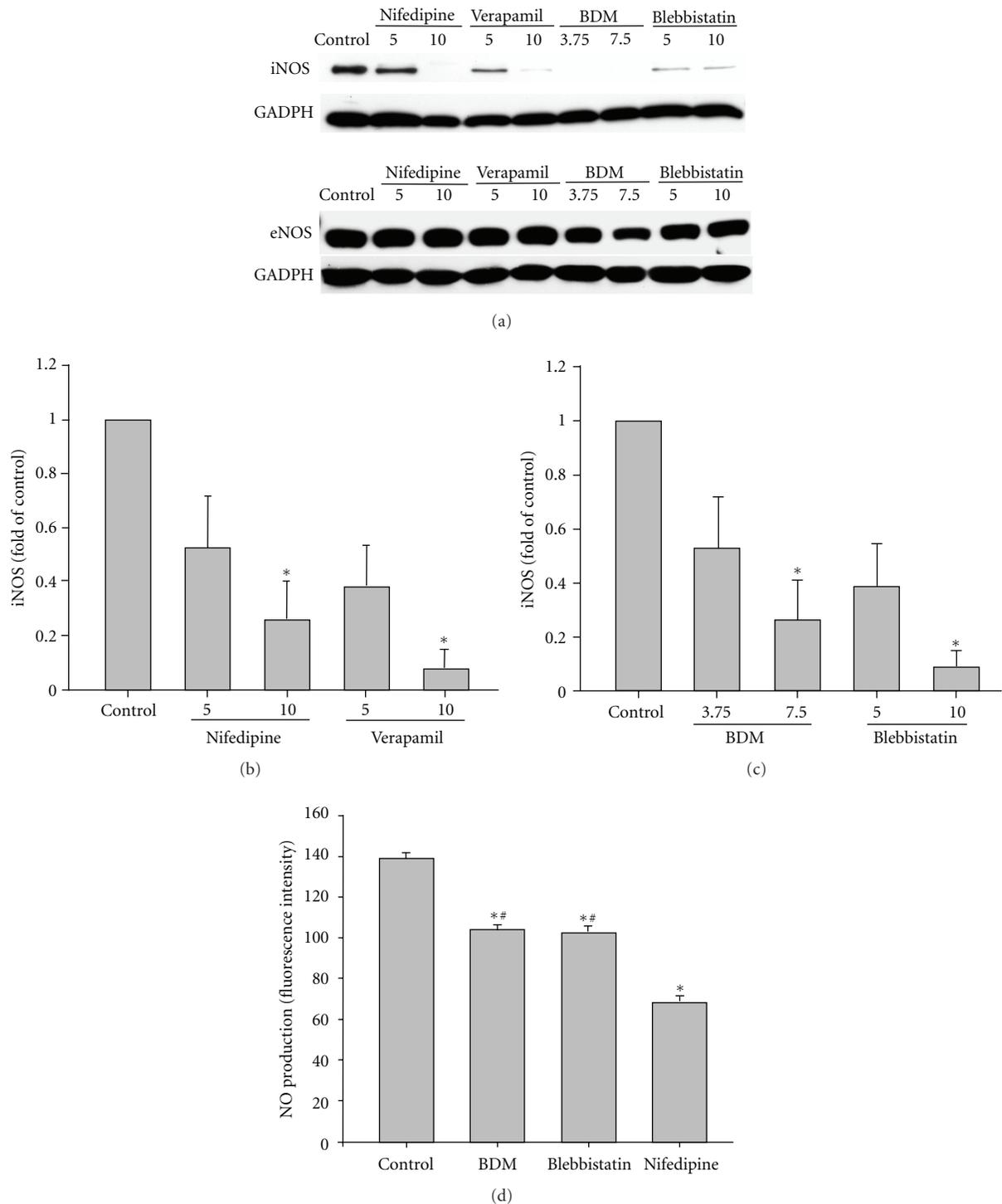


FIGURE 1: Contractile activity is essential for iNOS expression. (a) NRVMs were treated with L-type calcium channel blockers (nifedipine 5–10 μ M or verapamil 5–10 μ M) or myosin II ATPase inhibitors (butanedione monoxime (BDM) 3.75–7.5 mM or blebbistatin 5–10 μ M) for 24 h. Cell extracts (50 μ g total protein per lane) were then separated by SDS-PAGE and Western blotting with antibodies specific for iNOS, eNOS, and GAPDH. (b and c) The quantitative results of 5 experiments are depicted. * $P < 0.05$ versus untreated, control cells. (d) NO_i was measured under control conditions and in the presence of 7.5 mM BDM, 10 μ M blebbistatin or 10 μ M nifedipine. The quantitative results of 5 experiments are depicted. * $P < 0.05$ versus untreated, control cells. # $P < 0.05$ versus Nifedipine-treated cells.

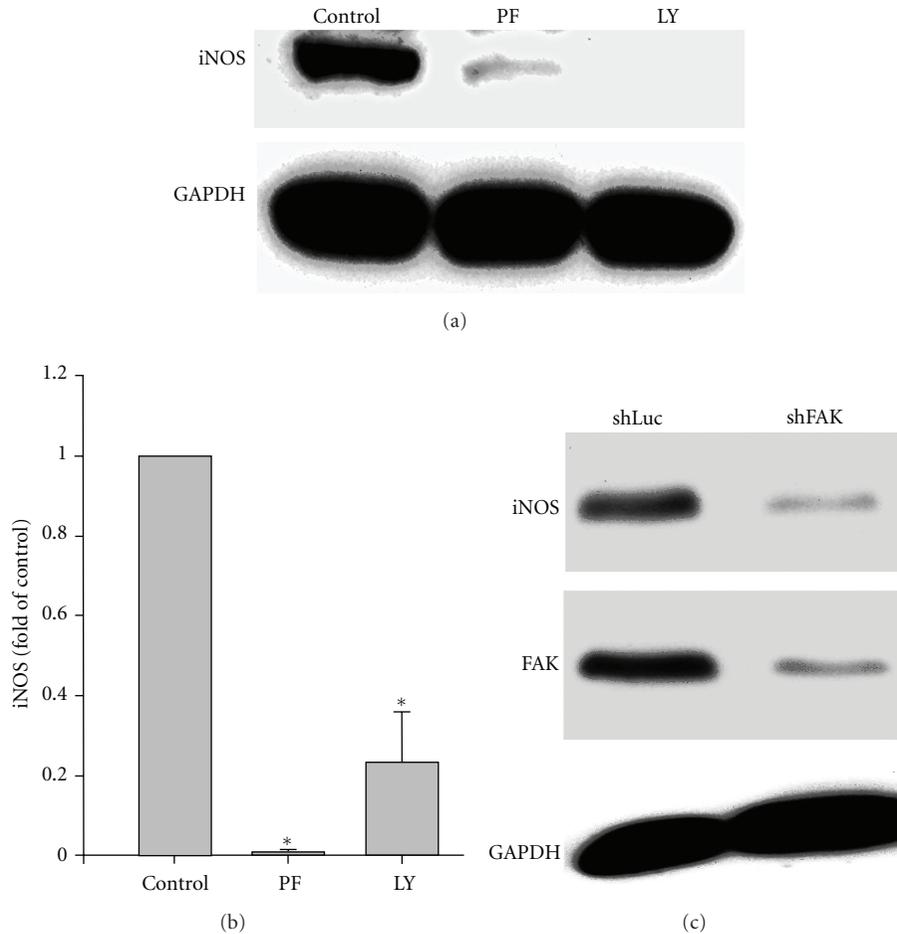


FIGURE 2: Contraction-induced iNOS expression is regulated by FAK and AKT. (a) NRVMs were maintained in control medium (con) or treated with PF 573228 (PF, 10 μ M) or LY 294002 (LY, 10 μ M) for 24 h. Cell extracts (50 μ g total protein per lane) were then separated by SDS-PAGE and Western blotting with antibodies specific for iNOS and GAPDH. (b) The quantitative results of 5 experiments are depicted. * $P < 0.05$ versus control. (c) NRVM were infected, (20 moi) with Adv-expressing shRNAs for luciferase (shLuc) or FAK (shFAK) and then maintained under control conditions for 72 h. Equal amounts of cell extracts (50 μ g total protein per lane) were then separated by SDS-PAGE and Western blotting with antibodies specific for FAK, iNOS, and GAPDH.

FAK knockdown also reduced AKT phosphorylation at both sites (Figure 3(c)). In contrast, shRNA-mediated knockdown of PYK2, the other member of the FAK family of protein tyrosine kinases, had much less of an effect on contraction-dependent AKT phosphorylation. Similar negative results were observed by overexpressing CRNK [42], the C-terminal inhibitor of PYK2 (data not shown). Finally, Adv-mediated overexpression of a “constitutively active” form of FAK (CD2-FAK) [21] was sufficient to further increase AKT phosphorylation in spontaneously contracting NRVMs (Figures 4(a) and 4(b)).

3.3. Rescue of iNOS Expression in Contractile-Arrested NRVMs. To further examine the role of FAK, PI(3)K, and AKT in regulating contraction-dependent iNOS expression, we attempted to “rescue” BDM inhibition of iNOS expression by overexpressing wildtype (WT) FAK, “constitutively

active” FAK (i.e., CD2-FAK), WT-AKT, or a constitutively active form of AKT (Myr-AKT) [22]. As seen in Figures 5(a) and 5(b), overexpression of WT-FAK had no effect on the BDM-mediated inhibition of iNOS expression. However, CD2-FAK overexpression rescued iNOS expression following BDM treatment. Of note, BDM treatment alone reduced iNOS expression to \sim 20% of spontaneously contracting NRVM. Overexpression of CD2-FAK in BDM-arrested NRVM caused a relative \sim 8-fold increase in iNOS expression, thus restoring its expression to levels above that observed in contracting cells (Figures 5(a) and 5(b)). Similarly, Myr-AKT overexpression also reversed the inhibitory effect of BDM (Figures 5(a) and 5(c)). To confirm these results, we performed the rescue experiment on BDM-treated cells but measured NO_i production using DAF-2-DA. Both WT-AKT and Myr-AKT were sufficient to restore NO_i production under BDM inhibition (Myr-AKT > WT-AKT) (Figure 5(d)). These results confirmed that FAK and AKT are

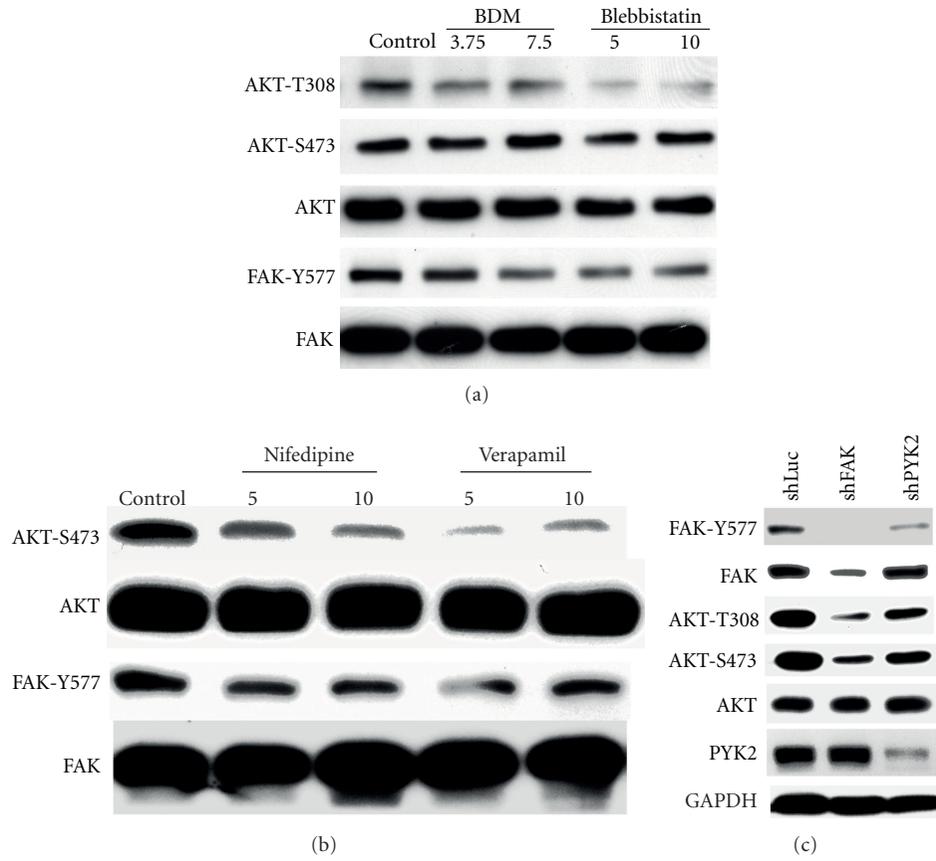


FIGURE 3: FAK regulates contraction-dependent activation of AKT. (a) NRVMs were treated with myosin II ATPase inhibitors (BDM or blebbistatin (Bleb)) or (b) L-type calcium channel blockers (nifedipine or verapamil) for 24 h. Cell extracts (50 μ g total protein per lane) were then separated by SDS-PAGE and Western blotting with antibodies specific for FAK phosphorylated at Y577, total FAK, AKT phosphorylated at T308 and S473, and total AKT. (c) NRVMs were infected, (20 moi) with Adv-expressing shRNAs for luciferase (shLuc), FAK (shFAK), or PYK2 (shPYK2) and then maintained under control conditions for 72 h. Equal amounts of cell extracts (50 μ g total protein per lane) were then separated by SDS-PAGE and Western blotting with antibodies specific for FAK phosphorylated at Y397, total FAK, AKT phosphorylated at T308 and S473, total AKT, total PYK2, and GAPDH.

both necessary and sufficient for contraction-mediated iNOS expression and NO_i production in NRVM.

4. Discussion

As demonstrated in this report and our previous study [14], cardiomyocyte contractile activity is an important factor that regulates NOSs and NO_i production via Ca²⁺-dependent and Ca²⁺-independent signaling pathways. As demonstrated in Figure 1, nifedipine reduced NO_i production to a greater extent than either BDM or blebbistatin. Previous studies have also shown that acute mechanical stimulation, such as, by sheer stress [43, 44] and static stretch [45] stimulate eNOS-dependent NO_i production in endothelial cells. A similar effect of acute mechanical loading on NO_i production was observed in cardiomyocytes [14, 31, 46, 47]. However, in these studies, the acute load-dependent phosphorylation of eNOS at S1177 was likely the responsible mechanism. Furthermore, eNOS activity can be regulated via activation

of PI(3)K-AKT or Ca²⁺-CaM signaling in different cell types [14, 44, 48]. In our study, the additive effect of inhibition of Ca²⁺ influx and contractile arrest led to a further reduction in NO_i production as compared to the inhibition of mechanical activity alone. These results are consistent with other observations that Ca²⁺-dependent and Ca²⁺-independent NOSs are both involved in mechanical stimulation-induced NO_i production. In our previous study, we showed that either decreased (BDM or blebbistatin) or increased (EMD 57033 or CGP 48506) contractile strength affected changes in field stimulation-induced NO_i production without changing Ca²⁺ influx (12). Here we demonstrate that contractile activity can also induce iNOS gene expression and NO_i production through a FAK-PI(3)K-AKT signaling pathway without interfering with Ca²⁺ handling. A schematic diagram outlining this signaling pathway is depicted in Figure 5(e).

Previous studies have shown that mechanical stretch can induce both iNOS and eNOS expression in NRVM [10, 49]. However in the present study, we could only detect

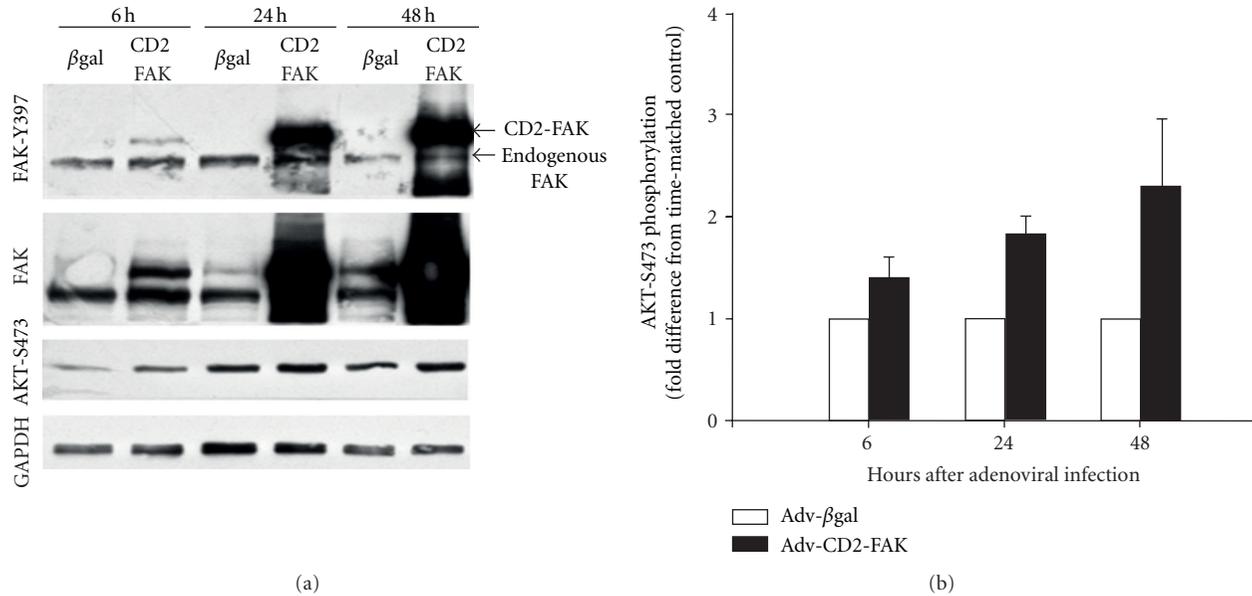


FIGURE 4: AKT activation is mediated by FAK. (a) NRVMs were infected (5 moi, 6–48 h) with Adv-expressing β gal or CD2-FAK. Cell extracts (50 μ g total protein per lane) were then separated by SDS-PAGE and Western blotting with antibodies specific for FAK phosphorylated at Y397, total FAK, AKT phosphorylated at S473, and total AKT. (b) The quantitative results of 9 experiments are depicted. * $P < 0.05$ versus time-matched, Adv- β gal cells.

a significant reduction in iNOS (but not eNOS) expression when cells were treated with L-type Ca^{2+} channel blockers or myosin II ATPase inhibitors. These results suggest that load-induced eNOS upregulation may be mediated by a different signaling mechanism. Liao et al. [10] also indicated that the stretch-induced Ca^{2+} signal was very likely the trigger for subsequent NO_i signaling in cardiomyocytes, and the NO_i signal further amplified itself by increasing iNOS expression. However, both types of inhibitors could not completely eliminate iNOS expression or NO_i production, suggesting that contraction-induced iNOS expression may also be subjected to NO_i autoregulation.

Our experiments utilized NRVM plated at high density ($\sim 1 \times 10^5$ cells/cm²) in order to enhance spontaneous contractile activity and increase iNOS expression. Under these conditions, FAK and AKT were also highly phosphorylated, but their phosphorylation could be reduced by inhibitors of contractile activity. Hines et al. [50] have also shown that dense myocyte cultures displayed higher metabolic activity and contraction rates as compared to cells plated at low density. Contractility may also regulate FAK, PI(3)K, and AKT activity in vivo. For instance, obese type 2 diabetic (db/db) mice have significantly reduced cardiac contractility and depressed PI(3)K-AKT signaling [51]. Conversely, acute pressure overload increased FAK, PI(3)K, and AKT activation in the intact heart [52]. These data suggest that cardiomyocyte contractility can activate several downstream signaling cascades in order to maintain cardiac function.

Although FAK appears both necessary and sufficient to induce AKT activation in NRVM, the intermediary steps

linking contractile activity to FAK and AKT activation remain unclear. As indicated in our previous paper, contraction-induced NO_i production required PI(3)K and AKT, as the highly specific PI(3)K inhibitor LY294002, or overexpression of a dominant-negative mutant of AKT, partially blocked contraction-dependent NO_i production [14]. Similar results were obtained by Del Re et al. [35], who showed that static stretch-induced AKT activation required FAK and PI(3)K. Furthermore, we recently showed that adhesion-dependent AKT activation in adult atrial myocytes was also dependent on FAK and PI(3)K [53]. In this regard, there are several potential mechanisms whereby PI(3)K may be involved. First, previous studies in nonmuscle cells have indicated that the p85 regulatory subunit of PI(3)K is tyrosine phosphorylated by FAK and can directly bind to the FAK-Y397 autophosphorylation site, thereby activating the lipid kinase [36–41]. Wei and Vander Heide [34] showed that FAK and p85 formed a complex in unstimulated NRVMs, and their association increased in response to heat stress. The heat stress-induced complexation of FAK and p85 was associated with an increase in AKT phosphorylation, which was prevented by FRNK overexpression. Del Re et al. [35] also demonstrated a similar static stretch-induced complexation of FAK and p85 in NRVM. Thus, a direct interaction between FAK and PI(3)K appears to be operative in cardiomyocytes.

Alternatively, Taniyama et al. [54] have shown that angiotensin II stimulated the tyrosine phosphorylation and activation of PDK1 in vascular smooth muscle cells via a PYK2- and c-Src-dependent pathway. Guo et al. [55] have shown that in NRVM, a similar pathway is activated by

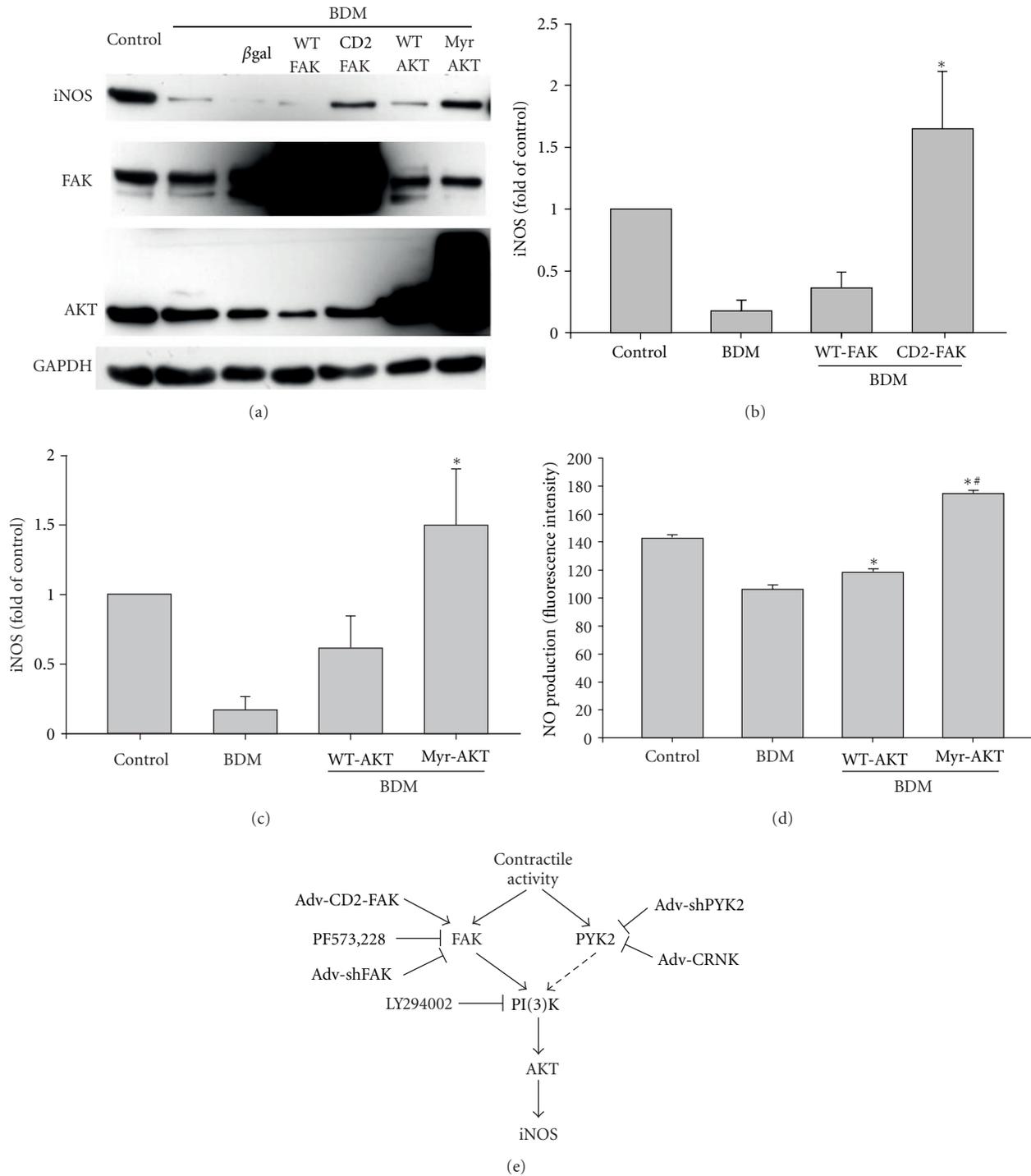


FIGURE 5: Rescue of BDM inhibition of iNOS expression by CD2-FAK and Myr-AKT. (a) NRVMs were pretreated with BDM (7.5 mM) for 24 h. To “rescue” the BDM inhibition of iNOS expression, some cells were then infected (48 h, 10 moi) with Adv-expressing β gal, WT-FAK, CD2-FAK, WT-AKT, or Myr-AKT with continued exposure to BDM. Cell extracts (50 μ g total protein per lane) were then separated by SDS-PAGE and Western blotting with antibodies specific for FAK, AKT, iNOS, and GAPDH. ((a) and (c)) The quantitative results of 5 experiments are depicted. * $P < 0.05$ versus WT-FAK or WT-AKT cells. (d) NRVMs were treated with 7.5 mM BDM for 24 h. To “rescue” the BDM inhibition of NO_i production, some cells were then infected (10 moi) with WT-AKT or Myr-AKT for 48 h with continued exposure to BDM. NO_i was then measured using DAF-2 DA. The quantitative results of 5 experiments are depicted. * $P < 0.05$ versus BDM, # $P < 0.05$ versus WT-AKT. (e) A multicomponent signaling pathway that links mechanical load to AKT activation and downstream signaling to iNOS is depicted.

H₂O₂, leading to downstream phosphorylation of AKT at both T308 and S473. Although we demonstrate that PYK2 is not necessary for contraction-induced AKT activation, it is conceivable that FAK, or the FAK/Src complex may function in a similar manner, thereby causing the tyrosine phosphorylation of PDK1 and subsequent AKT activation. Thus, the requirement for PI(3)K to mediate contraction-induced AKT activation would be conferred through the generation of phosphoinositides necessary for PDK1 and AKT recruitment to the cell membrane and not directly via formation of a FAK/PI(3)K complex. Of note, Xia et al. [41] demonstrated that ligation of β_1 integrins (accomplished with an anti- β_1 integrin antibody) activated both FAK and AKT in a Src- and PI(3)K-dependent manner, although FAK-p85 complexation could not be demonstrated.

Several studies have used genetically modified animal models to address the function of iNOS in the heart. Three different research groups have shown that iNOS deletion improved ventricular function, reduced myocardial nitrotyrosine content, decreased apoptosis, and improved survival after experimental myocardial infarction [56–58]. Zhang et al. [59] also demonstrated that iNOS deletion protected against pressure overload-induced ventricular hypertrophy and heart failure, in association with decreased myocardial nitrotyrosine and 4-hydroxy-2-nonenal. Furthermore, Mungrue et al. [60] have shown that cardiac-specific iNOS overexpression caused increased myocardial peroxynitrite production, myocardial fibrosis, ventricular hypertrophy, heart failure, and sudden cardiac death. These studies suggest that iNOS has significant detrimental effects on cardiac function. However, other studies have shown contradictory results, in that iNOS deficiency did not reduce myocardial infarct-induced ventricular dysfunction or mortality [61]. In fact, other studies demonstrated that iNOS plays a cardioprotective role during myocardial ischemia/reperfusion [62, 63]. Thus, the function of iNOS in the cardiomyocyte remains unclear, and additional studies are needed to evaluate the role of contractile activity-induced iNOS expression in regulating cardiomyocyte function and cell survival.

Acknowledgments

These studies were supported in part by NIH 2P01 HL62426, NIH 1F32 HL096143 and a grant from the Dr. Ralph and Marian Falk Medical Research Trust. Y. Koshman was also an AHA Postdoctoral fellow during the time these studies were performed.

References

- [1] J. L. Balligand, D. Ungureanu-Longrois, W. W. Simmons et al., "Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro," *Journal of Biological Chemistry*, vol. 269, no. 44, pp. 27580–27588, 1994.
- [2] K. Y. Xu, D. L. Huso, T. M. Dawson, D. S. Bredt, and L. C. Becker, "Nitric oxide synthase in cardiac sarcoplasmic reticulum," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 2, pp. 657–662, 1999.
- [3] O. Feron, L. Belhassen, L. Kobzik, T. W. Smith, R. A. Kelly, and T. Michel, "Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells," *Journal of Biological Chemistry*, vol. 271, no. 37, pp. 22810–22814, 1996.
- [4] L. A. Barouch, R. W. Harrison, M. W. Skaf et al., "Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms," *Nature*, vol. 416, no. 6878, pp. 337–339, 2002.
- [5] P. B. Massion, O. Feron, C. Dessy, and J. L. Balligand, "Nitric oxide and cardiac function: ten years after, and continuing," *Circulation Research*, vol. 93, no. 5, pp. 388–398, 2003.
- [6] S. Yamamoto, H. Tsutsui, H. Tagawa et al., "Role of myocyte nitric oxide in β -adrenergic hyporesponsiveness in heart failure," *Circulation*, vol. 95, no. 5, pp. 1111–1114, 1997.
- [7] O. Gealekman, Z. Abassi, I. Rubinstein, J. Winaver, and O. Binah, "Role of myocardial inducible nitric oxide synthase in contractile dysfunction and β -adrenergic hyporesponsiveness in rats with experimental volume-overload heart failure," *Circulation*, vol. 105, no. 2, pp. 236–243, 2002.
- [8] M. T. Ziolo, L. S. Maier, V. Piacentino III, J. Bossuyt, S. R. Houser, and D. M. Bers, "Myocyte nitric oxide synthase 2 contributes to blunted β -adrenergic response in failing human hearts by decreasing Ca²⁺ Transients," *Circulation*, vol. 109, no. 15, pp. 1886–1891, 2004.
- [9] N. Kobayashi, T. Higashi, K. Hara, H. Shirataki, and H. Matsuoka, "Effects of imidapril on NOS expression and myocardial remodelling in failing heart of Dahl salt-sensitive hypertensive rats," *Cardiovascular Research*, vol. 44, no. 3, pp. 518–526, 1999.
- [10] X. Liao, J. M. Liu, L. Du et al., "Nitric oxide signaling in stretch-induced apoptosis of neonatal rat cardiomyocytes," *The FASEB Journal*, vol. 20, no. 11, pp. 1883–1885, 2006.
- [11] R. Knöll, M. Hoshijima, and K. Chien, "Cardiac mechanotransduction and implications for heart disease," *Journal of Molecular Medicine*, vol. 81, no. 12, pp. 750–756, 2003.
- [12] A. M. Samarel, "Costameres, focal adhesions, and cardiomyocyte mechanotransduction," *American Journal of Physiology*, vol. 289, no. 6, pp. H2291–H2301, 2005.
- [13] B. P. Eliceiri, "Integrin and growth factor receptor crosstalk," *Circulation Research*, vol. 89, no. 12, pp. 1104–1110, 2001.
- [14] E. N. Dedkova, Y. G. Wang, X. Ji, L. A. Blatter, A. M. Samarel, and S. L. Lipsius, "Signalling mechanisms in contraction-mediated stimulation of intracellular NO production in rat ventricular myocytes," *Journal of Physiology*, vol. 580, no. 1, pp. 327–345, 2007.
- [15] A. M. Samarel and G. L. Engelmann, "Contractile activity modulates myosin heavy chain- β expression in neonatal rat heart cells," *American Journal of Physiology*, vol. 261, no. 4, pp. H1067–H1077, 1991.
- [16] Y. E. Koshman, M. Chu, S. J. Engman, T. Kim, R. Iyengar, S. L. Robia et al., "Focal adhesion kinase-related nonkinase inhibits vascular smooth muscle cell invasion by focal adhesion targeting, tyrosine 168 phosphorylation, and competition for p130^{Cas} binding," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, pp. 2432–2440, 2011.
- [17] M. C. Heidkamp, A. L. Bayer, J. A. Kalina, D. M. Eble, and A. M. Samarel, "GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes," *Circulation Research*, vol. 90, no. 12, pp. 1282–1289, 2002.

- [18] S. Sakurai, Y. Sonoda, E. Koguchi, N. Shinoura, H. Hamada, and T. Kasahara, "Mutated focal adhesion kinase induces apoptosis in a human glioma cell line, T98G," *Biochemical and Biophysical Research Communications*, vol. 293, no. 1, pp. 174–181, 2002.
- [19] A. Sorokin, P. Kozlowski, L. Graves, and A. Philip, "Protein-tyrosine kinase pyk2 mediates endothelin-induced p38^{MAPK} activation in glomerular mesangial cells," *Journal of Biological Chemistry*, vol. 276, no. 24, pp. 21521–21528, 2001.
- [20] D. M. Eble, J. B. Strait, G. Govindarajan, J. Lou, K. L. Byron, and A. M. Samarel, "Endothelin-induced cardiac myocyte hypertrophy: role for focal adhesion kinase," *American Journal of Physiology*, vol. 278, no. 5, pp. H1695–H1707, 2000.
- [21] P. Y. Chan, S. B. Kanner, G. Whitney, and A. Aruffo, "A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to PP125^{FAK}," *Journal of Biological Chemistry*, vol. 269, no. 32, pp. 20567–20574, 1994.
- [22] Y. Fujio, T. Nguyen, D. Wencker, R. N. Kitsis, and K. Walsh, "Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart," *Circulation*, vol. 101, no. 6, pp. 660–667, 2000.
- [23] M. C. Heidkamp, A. L. Bayer, J. L. Martin, and A. M. Samarel, "Differential activation of mitogen-activated protein kinase cascades and apoptosis by protein kinase C ϵ and δ in neonatal rat ventricular myocytes," *Circulation Research*, vol. 89, no. 10, pp. 882–890, 2001.
- [24] D. D. Schlaepfer and T. Hunter, "Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases," *Molecular and Cellular Biology*, vol. 16, no. 10, pp. 5623–5633, 1996.
- [25] H. Kojima, N. Nakatsubo, K. Kikuchi et al., "Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins," *Analytical Chemistry*, vol. 70, no. 13, pp. 2446–2453, 1998.
- [26] N. Nakatsubo, H. Kojima, K. Kikuchi et al., "Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins," *FEBS Letters*, vol. 427, no. 2, pp. 263–266, 1998.
- [27] E. N. Dedkova, Y. G. Wang, L. A. Blatter, and S. L. Lipsius, "Nitric oxide signalling by selective β_2 -adrenoceptor stimulation prevents ACh-induced inhibition of β_2 -stimulated Ca^{2+} current in cat atrial myocytes," *Journal of Physiology*, vol. 542, no. 3, pp. 711–723, 2002.
- [28] K. L. Byron, J. L. Puglisi, J. R. Holda, D. Eble, and A. M. Samarel, "Myosin heavy chain turnover in cultured neonatal rat heart cells: effects of $[\text{Ca}^{2+}]_i$ and contractile activity," *American Journal of Physiology*, vol. 271, no. 5, pp. C1447–C1456, 1996.
- [29] M. Qi, J. L. Puglisi, K. L. Byron et al., "Myosin heavy chain gene expression in neonatal rat heart cells: effects of $[\text{Ca}^{2+}]_i$ and contractile activity," *American Journal of Physiology*, vol. 273, no. 2, pp. C394–C403, 1997.
- [30] D. M. Eble, M. Qi, S. Waldschmidt, P. A. Lucchesi, K. L. Byron, and A. M. Samarel, "Contractile activity is required for sarcomeric assembly in phenylephrine-induced cardiac myocyte hypertrophy," *American Journal of Physiology*, vol. 274, no. 5, pp. C1226–C1237, 1998.
- [31] M. G. V. Petroff, S. H. Kim, S. Pepe et al., "Endogenous nitric oxide mechanisms mediate the stretch dependence of Ca^{2+} release in cardiomyocytes," *Nature Cell Biology*, vol. 3, no. 10, pp. 867–873, 2001.
- [32] J. K. Slack-Davis, K. H. Martin, R. W. Tilghman et al., "Cellular characterization of a novel focal adhesion kinase inhibitor," *Journal of Biological Chemistry*, vol. 282, no. 20, pp. 14845–14852, 2007.
- [33] C. J. Vlahos, W. F. Matter, K. Y. Hui, and R. F. Brown, "A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)," *Journal of Biological Chemistry*, vol. 269, no. 7, pp. 5241–5248, 1994.
- [34] H. Wei and R. S. Vander Heide, "Heat stress activates AKT via focal adhesion kinase-mediated pathway in neonatal rat ventricular myocytes," *American Journal of Physiology*, vol. 295, no. 2, pp. H561–H568, 2008.
- [35] D. P. Del Re, S. Miyamoto, and J. H. Brown, "Focal adhesion kinase as a RhoA-activable signaling scaffold mediating akt activation and cardiomyocyte protection," *Journal of Biological Chemistry*, vol. 283, no. 51, pp. 35622–35629, 2008.
- [36] H. C. Chen and J. L. Guan, "Stimulation of phosphatidylinositol 3'-kinase association with focal adhesion kinase by platelet-derived growth factor," *Journal of Biological Chemistry*, vol. 269, no. 49, pp. 31229–31233, 1994.
- [37] H. C. Chen and J. L. Guan, "Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 21, pp. 10148–10152, 1994.
- [38] C. Bachelot, L. Rameh, T. Parsons, and L. C. Cantley, "Association of phosphatidylinositol 3-kinase, via the SH2 domains of p85, with focal adhesion kinase in polyoma middle t-transformed fibroblasts," *Biochimica et Biophysica Acta*, vol. 1311, no. 1, pp. 45–52, 1996.
- [39] H. C. Chen, P. A. Appeddu, H. Isoda, and J. L. Guan, "Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase," *Journal of Biological Chemistry*, vol. 271, no. 42, pp. 26329–26334, 1996.
- [40] H. R. Reiske, S. C. Kao, L. A. Cary, J. L. Guan, J. F. Lai, and H. C. Chen, "Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration," *Journal of Biological Chemistry*, vol. 274, no. 18, pp. 12361–12366, 1999.
- [41] H. Xia, R. S. Nho, J. Kahm, J. Kleidon, and C. A. Henke, "Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a β_1 integrin viability signaling pathway," *Journal of Biological Chemistry*, vol. 279, no. 31, pp. 33024–33034, 2004.
- [42] D. L. Hart, M. C. Heidkamp, R. Iyengar et al., "CRNK gene transfer improves function and reverses the myosin heavy chain isoenzyme switch during post-myocardial infarction left ventricular remodeling," *Journal of Molecular and Cellular Cardiology*, vol. 45, no. 1, pp. 93–105, 2008.
- [43] S. Dimmeler, I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, and A. M. Zeiher, "Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation," *Nature*, vol. 399, no. 6736, pp. 601–605, 1999.
- [44] D. Fulton, J. P. Gratton, T. J. McCabe et al., "Regulation of endothelium-derived nitric oxide production by the protein kinase Akt," *Nature*, vol. 399, no. 6736, pp. 597–601, 1999.
- [45] W. M. Kuebler, U. Uhlir, T. Goldmann et al., "Stretch activates nitric oxide production in pulmonary vascular endothelial cells in situ," *American Journal of Respiratory and Critical Care Medicine*, vol. 168, no. 11, pp. 1391–1398, 2003.
- [46] D. J. Pinsky, S. Patton, S. Mesaros et al., "Mechanical transduction of nitric oxide synthesis in the beating heart," *Circulation Research*, vol. 81, no. 3, pp. 372–379, 1997.

- [47] B. D. Prendergast, V. F. Sagach, and A. M. Shah, "Basal release of nitric oxide augments the Frank-Starling response in the isolated heart," *Circulation*, vol. 96, no. 4, pp. 1320–1329, 1997.
- [48] I. Fleming, B. Fisslthaler, S. Dimmeler, B. E. Kemp, and R. Busse, "Phosphorylation of Thr⁴⁹⁵ regulates Ca²⁺/calmodulin-dependent endothelial nitric oxide synthase activity," *Circulation Research*, vol. 88, no. 11, pp. E68–E75, 2001.
- [49] T. H. Cheng, J. J. W. Chen, N. L. Shih et al., "Mechanical stretch induces endothelial nitric oxide synthase gene expression in neonatal rat cardiomyocytes," *Clinical and Experimental Pharmacology and Physiology*, vol. 36, no. 5-6, pp. 559–566, 2009.
- [50] W. A. Hines, J. Thorburn, and A. Thorburn, "Cell density and contraction regulate p38 MAP kinase-dependent responses in neonatal rat cardiac myocytes," *American Journal of Physiology*, vol. 277, no. 1, pp. H331–H341, 1999.
- [51] N. D. Roe and J. Ren, "Akt2 knockout mitigates chronic iNOS inhibition-induced cardiomyocyte atrophy and contractile dysfunction despite persistent insulin resistance," *Toxicology Letters*, vol. 207, pp. 222–231, 2011.
- [52] K. G. Franchini, A. S. Torsoni, P. H. A. Soares, and M. J. A. Saad, "Early activation of the multicomponent signaling complex associated with focal adhesion kinase induced by pressure overload in the rat heart," *Circulation Research*, vol. 87, no. 7, pp. 558–565, 2000.
- [53] Y. G. Wang, X. Ji, M. Pabbidi, A. M. Samarel, and S. L. Lipsius, "Laminin acts via focal adhesion kinase/phosphatidylinositol-3'kinase/protein kinase B to down-regulate β_1 -adrenergic receptor signalling in cat atrial myocytes," *Journal of Physiology*, vol. 587, no. 3, pp. 541–550, 2009.
- [54] Y. Taniyama, D. S. Weber, P. Rocic et al., "Pyk2- and src-dependent tyrosine phosphorylation of PDK1 regulates focal adhesions," *Molecular and Cellular Biology*, vol. 23, no. 22, pp. 8019–8029, 2003.
- [55] J. Guo, A. Sabri, H. Elouardighi, V. Rybin, and S. F. Steinberg, " α_1 -adrenergic receptors activate AKT via a Pyk2/PDK-1 pathway that is tonically inhibited by novel protein kinase C isoforms in cardiomyocytes," *Circulation Research*, vol. 99, no. 12, pp. 1367–1375, 2006.
- [56] Q. Feng, X. Lu, D. L. Jones, J. Shen, and J. M. O. Arnold, "Increased inducible nitric oxide synthase expression contributes to myocardial dysfunction and higher mortality after myocardial infarction in mice," *Circulation*, vol. 104, no. 6, pp. 700–704, 2001.
- [57] F. Sam, D. B. Sawyer, Z. Xie et al., "Mice lacking inducible nitric oxide synthase have improved left ventricular contractile function and reduced apoptotic cell death late after myocardial infarction," *Circulation Research*, vol. 89, no. 4, pp. 351–356, 2001.
- [58] Y. H. Liu, O. A. Carretero, O. H. Cingolani et al., "Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction," *American Journal of Physiology*, vol. 289, no. 6, pp. H2616–H2623, 2005.
- [59] P. Zhang, X. Xu, X. Hu, E. D. van Deel, G. Zhu, and Y. Chen, "Inducible nitric oxide synthase deficiency protects the heart from systolic overload-induced ventricular hypertrophy and congestive heart failure," *Circulation Research*, vol. 100, no. 7, pp. 1089–1098, 2007.
- [60] I. N. Mungrue, R. Gros, X. You et al., "Cardiomyocyte overexpression of iNOS in mice results in peroxynitrite generation, heart block, and sudden death," *Journal of Clinical Investigation*, vol. 109, no. 6, pp. 735–743, 2002.
- [61] S. P. Jones, J. J. M. Greer, P. D. Ware, J. Yang, K. Walsh, and D. J. Lefer, "Deficiency of iNOS does not attenuate severe congestive heart failure in mice," *American Journal of Physiology*, vol. 288, no. 1, pp. H365–H370, 2005.
- [62] R. Bolli, "Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 11, pp. 1897–1918, 2001.
- [63] S. P. Jones and R. Bolli, "The ubiquitous role of nitric oxide in cardioprotection," *Journal of Molecular and Cellular Cardiology*, vol. 40, no. 1, pp. 16–23, 2006.



Hindawi

Submit your manuscripts at
<http://www.hindawi.com>

