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Cardioprotective Stimuli Mediate Phosphoinositide 3-Kinase and Phosphoinositide Dependent Kinase 1 Nuclear Accumulation in Cardiomyocytes

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Abstract

The phosphoinositide-3-kinase (PI3K) / phosphoinositide dependent kinase 1 (PDK1) signaling pathway exerts cardioprotective effects in the myocardium through activation of key proteins including Akt. Activated Akt accumulates in nuclei of cardiomyocytes suggesting that biologically relevant targets are located in that subcellular compartment. Nuclear Akt activity could be potentiated in both intensity and duration by the presence of a nuclear-associated PI3K / PDK1 signaling cascade as has been described in other non-myocyte cell types. PI3K / PDK1 distribution was determined *in vitro* and *in vivo* by immunostaining and nuclear extraction of cultured rat neonatal cardiomyocytes or transgenic mouse hearts. Results show that PI3K and PDK1 are present at a basal level in cardiomyocytes nuclei and that cardioprotective stimulation with atrial natriuretic peptide (ANP) increases their nuclear localization. In comparison, overexpression of nuclear-targeted Akt does not mediate increased translocation of either PI3K or PDK1 indicating that accumulation of Akt does not drive PI3K or PDK1 into the nuclear compartment. Furthermore, PI3K and phospho-Akt⁴⁷³ show parallel temporal accumulation in the nucleus following (MI) infarction challenge. These findings demonstrate the presence of a dynamically regulated nuclear-associated signaling cascade involving PI3K and PDK that presumably influences nuclear Akt activation.

Keywords

Nucleus; PI3K; PDK1; cardiomyocyte; Akt; phosphoinositide

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Introduction

Phosphoinositide 3-kinase (PI3K) dependent signaling is a ubiquitous pathway involved in cell growth, proliferation, survival, migration, metabolism and several other biological responses [1]. Class I PI3Ks are activated by receptor tyrosine kinase/cytokine receptor activation (class I_A, PI3K α , β and δ) or by G-protein coupled receptors (Class I_B, PI3K γ), leading to increased phosphatidylinositol (3,4,5) triphosphate (PIP₃) levels. Under basal conditions PDK1 and AKT form complexes both in the cytoplasm and at the membrane that is in continuous equilibrium between associated and dissociated forms. AKT inactivity is maintained by interaction between PH domain and kinase domains [2]. Pursuant to receptor stimulation, elevation of PIP₃ level induces concentration of molecules possessing PH domains at the plasma membrane promoting interaction between PDK1 and AKT resulting in allowing PDK1 to phosphorylate AKT at the T-loop Thr³⁰⁸ residue [3,4]. Activated AKT remains in activated form upon dissociation from the plasma membrane and accumulates both in the cytoplasm and the nucleus [2]. A second phosphorylation within a C-terminal hydrophobic motif at Ser⁴⁷³ is mediated by the mTOR complex 2 (mTORC2) empowering AKT to be fully activated [5,6]. Activated Akt is then able to phosphorylate members of the FOXO class of transcription factors and induce their segregation from the nuclear compartment [7].

Upon physiological stimuli, Akt is activated and accumulates in the nucleus of cardiomyocytes [8,9] preventing apoptosis induced by different kind of pathological insults including ischemia reperfusion [10,11], volume and/or pressure overload, hypoxia [12], hypoglycemia, or cardiotoxic drugs [13]. Thus, the net result of PI3K activity is enhanced nuclear accumulation of Akt that impacts cardiomyocyte survival, remodeling, and myocardial proliferation [8,14, 15]. However constitutive AKT activation resulting from adenoviral gene transfer or transgenic expression induces hypertrophy in cardiomyocytes through phosphorylation of target substrate molecules which are predominantly located in the cytoplasm and leads to hypertrophic cardiomyopathy *in vivo* [16,17].

Cellular targets of Akt mediating pro-proliferative or anti-apoptotic effects reside within the nuclear compartment [18-20]. Effects of nuclear Akt signaling have been studied by our group through use of a modified Akt expressed in wild-type (non-activated) form targeted to the nucleus by incorporation of a nuclear localization signal (Akt-nuc)[8]. Akt-nuc exerts significant cardioprotective, proliferative, and inotropic effects in cardiomyocytes [15,21] without induction of hypertrophic response both *in vitro* and *in vivo* [14]. Akt-nuc shows these biological activities despite being targeted directly to the nucleus rather than the conventional paradigm of membrane-associated activation and subsequent nuclear accumulation [1]. To explain the inherent activity of Akt-nuc, we hypothesized that a nuclear PI3K / PDK1 signaling network was present in cardiomyocytes and that could be augmented in response to cardioprotective stimulation. Results of our studies reveal that PI3K and PDK1 are present in the nuclei of cardiomyocytes, both in primary neonatal cultures and adult hearts, and that nuclear levels are enhanced upon stimulation by atrial natriuretic peptide (ANP) at anti-apoptotic concentration. Furthermore, PI3K and phospho-Akt⁴⁷³ show parallel temporal accumulation in the nucleus, suggesting the existence of a regulated nuclear PI3K/PDK1 signaling cascade as a previously unrecognized mechanism for increasing nuclear Akt signal intensity and duration in cardiomyocytes following cardioprotective stimulation or cardiomyopathic challenge [8].

Materials and Methods

Mice

All animal protocols have been approved by the Institutional Animal Care Committee of San Diego State University. Hearts were fixed and paraffin embedded or collected for nuclear fractionation. Infarction studies were performed as described in the supplement.

Neonatal rat cardiomyocyte isolation and culture

Neonatal rat ventricular myocytes were isolated as previously described [22] Details of methods are provided in the supplementary section.

Nuclear fractionation of heart tissue and cultured cells

Nuclear fractions were obtained as described before by Camper-Kirby *et al.* [9]. with a slight modification for cultured cells. Some experiments were performed using an alternative protocol. Details of the methods are reported in in the supplementary section.

Immunostaining and microscopy

Details of procedures for immunolabeling of cultured neonatal rat cardiomyocytes and paraffin sections are provided in the Supplementary methods. Nuclear accumulation in myocardial sections was quantitated as the percentage of positive nuclei per total number of cardiomyocytes observed. Colocalization quantitations were calculated using CoLocalizer Pro version 1.2 (CoLocalization Research Software) analysis software with at least 1000 cells counted in each experiment.

Western blotting

Western blotting was performed using standard techniques as described in the supplementary section.

Statistics

Statistical analysis was performed on at least 3 independent observations in each experimental set by 1-way analysis of variance (ANOVA) or Student's *t* test, according to the experimental design. If the overall ANOVA *p* value was significant, pairwise comparisons were performed by Student-Newman-Keuls test. The GraphPad Prism software (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com) was used for computer analysis. The results are expressed as mean $\pm$ standard error of the mean (SEM). The threshold for statistical significance was set as $P < 0.05$.

Results

ANP stimulation promotes nuclear accumulation of PI3K and PDK1 in cultured neonatal rat cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes (NRCMs) were treated with ANP at 10^{-9} M, a concentration previously shown to mediate nuclear accumulation of Akt [22]. Localization of PI3K and PDK1 were determined by immunofluorescence of cells stained with antibodies to PI3Kp110 α (PI3K; catalytic subunit) and to PDK1. Nuclear accumulation was quantified as the percentage of cardiomyocytes with intense nuclear staining per total cardiomyocytes counted ($n=3$ independent experiments). Accumulation of PI3K in the nuclear compartment increased by 15 to 30 minutes, declining to basal levels after 45 minutes of ANP treatment (Figure 1A and 1B). Under the same conditions PDK1 nuclear accumulation peaked at 30 minutes and returned to basal levels by 60 minutes of treatment (Figure 2A - B). In order to

estimate the amount of nuclear translocation of the two kinases induced by ANP, western blots were performed on nuclear and cytosolic fractions of treated and non-treated cells. As observed by immunostaining, PI3K and PDK1 showed similar nuclear accumulation kinetics, increasing significantly compared to control at 30 minutes of ANP 10^{-9} M treatment as previously shown for Akt [22]. Phospho-Akt levels were concomitantly increased by ANP treatment (Figure 3A - B). Since changes in cytosolic levels for PI3K, PDK1 and phospho-Akt kinase were undetectable at this time (Figure 3B), presumably a sub-fraction of the available cytosolic protein pool is concentrated in the nuclear compartment upon stimulation. Further investigations by immunostaining and immunoblot confirmed the previously described increases of nuclear phospho-Akt^{T308} and phospho-Akt^{S473} following ANP treatment. Kinetics of accumulation were similar to that observed for PI3K and PDK1, peaking at 1 hour post treatment (Supplemental Figure 1).

Acute induction of PI3K and PDK1 nuclear localization is mediated by cGMP

Signaling leading to Akt activation lies downstream of an ANP-dependent signaling pathway that is mediated by cGMP [22]. Therefore, robust nuclear accumulation of Akt was induced in the myocardium via cGMP treatment of normal mice to corroborate findings of nuclear PI3K and PDK1 accumulation observed using cultured cardiomyocytes (Figures 1 - 3). Nuclear localization of PI3K and PDK1 increased by 98% and 64%, respectively, compared to vehicle injected controls at two hours after injection of cGMP (Supplemental Figure 2A-C). Immunoblot analysis of heart nuclear extracts from cGMP treated mice confirmed these results showing 59% and 24% increases in accumulation of PI3K and PDK1, respectively. No changes were observed in cytosolic fractions (Supplemental Figure 2D).

Nuclear-targeted Akt (Akt-nuc) does not promote higher levels of nuclear PI3K or PDK1

We have previously shown that a wild type Akt construct designed with a nuclear localization sequence becomes activated in the presumed absence of conventional cell membrane translocation to promote PIP₃ and PDK-1 interaction [8]. Multiple studies with our Akt-nuc construct show the kinase is functional and exerts potent cardioprotective effects, leaving the issue as to how Akt-nuc achieves active status without cytoplasmic membrane association unresolved. To address the potential influence of overexpressed Akt-nuc upon endogenous PI3K / PDK1 localization allowing for activation, NRCMs were infected with adenoviruses expressing either α -actinin-GFP as control or myc tagged Akt-nuc. Myocytes were fixed and stained for PI3K as well as myc-tag to detect the presence of Akt-nuc at 24 hours post infection. As shown in Supplemental Figure 3A-D, expression of Akt-nuc did not increase nuclear localization of PI3K or PDK1. Although viral infection induces low level PI3K nuclear translocation, no differences were observed between α -actinin-GFP control or Akt-nuc infected NRCMs within nuclear or cytosolic fractions (Supplemental Figure 3E). Immunoblots for PI3K performed on nuclear and cytosolic fractions corroborated staining results. The effect of recombinant adenoviruses expressing dominant negative Akt (Akt-DN) as well as wild-type Akt protein (Akt-wt) on nuclear accumulation of PI3K and PDK1 was also studied in NRCM cultures. Akt-wt-mediated effects were similar to Akt-nuc and did not increase nuclear localization of PI3K or PDK1 (Supplemental Figure 4).

Transgenic mice with cardiac-specific expression of Akt-nuc were examined to support and extend *in vitro* findings regarding PI3K and PDK1 localization. Nuclei from Akt-nuc hearts showed PI3K levels comparable to that of nontransgenic controls (NTG; Figure 4). However, a marked increase in nuclear PI3K was induced in Akt-nuc mice following intravenous cGMP treatment. Immunohistochemistry of Akt-nuc hearts from mice treated with cGMP showed an increase of myocytes possessing nuclear PI3K labeling relative to control samples treated with vehicle alone (Figure 5A - B). Immunoblots probing myocardial nuclear fractions for PI3K corroborate immunostaining, showing a significant increase in nuclear PI3K levels and no

change in cytosolic levels (Figure 5C bottom panel). Thus, forced nuclear accumulation of Akt does not influence nuclear accumulation PDK / PI3K accumulation.

Myocardial infarction promotes nuclear localization of PI3K concurrent with phospho-Akt⁴⁷³

Myocardial infarction (MI) provokes a reactive survival response in the border zone (BZ) surrounding the damaged tissue. Involvement of PI3K in this reactive signaling response was investigated in mice subjected to myocardial infarction (MI) by coronary artery ligation. Sections were stained to observe PI3K localization in the BZ surrounding the infarct area at 3 and 7 days post surgery. Infarcted myocardium was identified by loss of tropomyosin labeling (Figure 6B-C, infarcted zone, IZ). Increased PI3K nuclear localization was observed within the BZ in both myocytes and non-myocytes (Figures 6A-C, yellow arrows in overlays). Companion serial sections labeled for phospho-Akt⁴⁷³ show nuclear accumulation indicative of Akt activation (Figure 6A-C, right side of panels). In comparison, healthy myocardium of sham operated mice show typical staining for PI3K along myocyte striations with sporadic nuclear staining (Figure 6A).

Quantitative localization analysis for either PI3K or phospho-Akt^{S473} shows parallel trends in nuclear accumulation with significant increases at both three and seven days after MI (Figure 6D). In sham operated mice the percentage of cells showing PI3K nuclear staining was $9.04\% \pm 1.83$ that remained unchanged throughout the time course of the studies. In comparison, nuclear PI3K immunolabeling increases following MI, increasing at day 3 to $19.50\% \pm 1.60$ and at day 7 to $17.59\% \pm 3.57$. The percentage of cells showing nuclear pS473Akt was higher, with a basal level of $22.25\% \pm 3.65$ in sham operated mice that increases after MI to $48.74\% \pm 2.08$ at day 3 and to $61.75\% \pm 6.1$ at day 7.

Discussion

Recently the paradigm of phospholipids signaling primarily at the plasma membrane has shifted to incorporate an independent nuclear phospholipid cycle [23-25] as well as plasma membrane-associated events being initiated by a nuclear phosphoinositide signaling cascade [26-28]. Nuclear localization of components of the PI3K signaling pathway was first reported in 1994 when PI3K was found to translocate to the nucleus of PC12 cells upon nerve growth factor treatment [26]. Following this initial report, similar results were described in various cell types (HepG2 cells, osteoblast-like MC3T3-E1 cells and others) after stimulation by insulin, insulin-like growth factor 1 (IGF-1) and platelet derived growth factor [29-31]. PI3K is not the only component of this pathway that has been described to shuttle or exist in the nucleus; PDK1, PTEN and Akt have also been shown to translocate to the nucleus upon stimulation or to reside in the nucleus under basal [27,28,32].

In the heart, PI3K α , γ and PTEN are expressed and involved in modulating survival/apoptosis, hypertrophy metabolism and mechanotransduction. Several transgenic and knockout mouse models illustrate the fundamental role of the PI3K pathway in the regulation of myocardial contractility, hypertrophy and cell survival/apoptosis [33]. Cardiac specific overexpression of a constitutively active form of PI3K α in mice increased heart size via cardiomyocyte enlargement [34], whereas overexpression of a dominant negative form of PI3K α in the heart decreases heart size with a normal number of smaller myocytes [35]. Gene targeting of the PI3K γ isoform resulted in increased cardiac contractility without hypertrophy, and a muscle specific deletion of PTEN showed increased heart and cardiomyocyte size with reduced myocardial contractility [35]. A central role for PDK1 in the cardiac PI3K pathway was confirmed by a cardiac specific genetic deletion exhibiting decreased cardiomyocyte size, heart failure and increased sensitivity to hypoxia [36]. Among the primary downstream effectors of the PI3K pathway is Akt, which controls a variety of responses in the heart, such as inhibition

of apoptosis, regulation of cell proliferation, metabolism and hypertrophy. Akt targets including glycogen synthase kinase-3 β (GSK-3 β) [37], mammalian target of rapamycin (mTOR) [38], endothelial nitric oxide synthase (eNOS or NOS3) [39,40], 6-phospho-fructo-2-kinase (PFK2) [41], c-Raf (a serine/threonine kinase) [42] and several anti-apoptotic effectors [43-47] are localized throughout the cell in cytoplasmic, mitochondrial and nuclear compartments. PI3K activation via Akt enhances cell survival and antagonizes apoptosis in cardiomyocytes [48-50]. Consistent with the regulatory properties of Akt, Akt1 deletion results in mice that are smaller in size and have increased apoptosis [51,52], while mice overexpressing Akt in the heart display several phenotypes depending on the design used to express the kinase. Three different approaches to overexpress constitutively active Akt in the heart resulted in hypertrophy with reduced [16], normal [17] or enhanced [53] contractility. All of these approaches have a common feature of predominant membrane-cytoplasmic localization of the overexpressed kinase.

The present study demonstrates that two immediate upstream kinases necessary for Akt activation, PI3K and PDK1, are present in the nucleus of primary cultured neonatal rat cardiomyocytes and in adult mouse cardiac cells. These results extend similar findings from other cell types where most components of the PI3K pathway have been found to be inhabitants of the nucleus or commuters through that compartment [54,55].

The idea of an autonomous PI3K-Akt pathway present in the nucleus of cardiomyocytes under normal conditions, or being recruited after stimulation, adds another level of regulation for the phosphorylation of Akt and its targets in the nucleus. Several studies have shown that Akt phosphorylation is not required for nuclear translocation [56-58], which makes the idea of nuclear activation of Akt even more appealing [59]. In our study, ANP treatment causes PI3K and PDK1 nuclear accumulation peaking earlier in time (30 minutes) (Figure 1-3) than nuclear phospho-Akt (1h) (Supplementary figures 1-3) making it plausible for Akt activation to happen, at least in part, in the nucleus. This temporal pattern of migration to the nucleus has also been shown in MC3T3-E1 mouse calvaria fibroblasts, where IGF-1 and platelet derived growth factor stimulate an increase in nuclear active Akt preceded in time by translocation of PI3K to the nucleus [30,60].

The mechanism by which PI3K and/or PDK1 enter the nucleus remains obscure, even in non-cardiac cells where these events have been well documented. To date, none of the published literature has temporally separated the nuclear accumulation of both these kinases to examine their relationship, but the literature points to a co-dependence since treatment with PI3K inhibitor LY294002 that inactivates PI3K, leads to lack of PDK1 nuclear accumulation [61]. Nuclear-targeted Akt in the heart has been established to have a protective effect not only *in vitro* but also *in vivo* [8,15,21,62]. The fact that Akt-nuc needs to be phosphorylated in order to be active and exert its beneficial role, makes the existence of a nuclear independent activation system in cardiomyocytes (PI3K and PDK1) of key relevance. The presence of PI3K and PDK1 in the nucleus ensures activation of exogenously expressed Akt-nuc and provides a mechanistic basis to explain the activation of nuclear Akt in the absence of the traditional plasma membrane targeting or localization approaches. Phosphatase action represents another possible facet in the regulation of nuclear Akt activity, especially considering that PTEN has already been placed in the nucleus of several types of cells [55]. The relevance of phosphatases in modulation of nuclear Akt awaits future studies that will also delineate the relationship of this nuclear autonomous system in relation to the cytoplasmic compartment to determine which facets of Akt-mediated effects reside within these distinct cellular compartments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Disclosures: Non

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Figure 1. ANP stimulation promotes nuclear accumulation of PI3K in neonatal rat cardiomyocytes
 A) Representative confocal micrographs showing increased nuclear accumulation of PI3K into NRCMs nuclei. Lanes from top to bottom, show cells treated with ANP 10^{-9} M for increasing amount of time (0 to 60 minutes). Time 0 was considered as the baseline levels of PI3K into the nuclei (unstimulated myocytes). Columns from left to right represent PI3K (green), nuclei (blue), and actin filaments (red). Cardiomyocytes with increased nuclear staining of PI3K are shown and indicated by yellow arrows B) Quantification analysis of the time course showed in panel A. PI3K nuclear accumulation peaks at 30 minutes and decreased at control levels by 60 minutes. Scale bars: 40 μ m for panel A and 20 μ m for panel B (n=4; ANOVA $P<0.05$; *

$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. 0 minutes calculated by Student-Newman-Keuls post hoc test).

Figure 2. ANP stimulation promotes nuclear accumulation of PDK1 in neonatal rat cardiomyocytes
 A) Representative confocal micrographs showing increased nuclear accumulation of PDK1 into NRCMs nuclei. Lanes from top to bottom, represent cells treated with ANP 10^{-9} M for increasing amount of time (0 to 60 minutes). Time 0 was considered as the baseline levels of PDK1 into the nuclei (unstimulated myocytes). Columns from left to right represent PDK1 (green), nuclei (blue), and actin filaments (red). Cardiomyocytes with increased nuclear staining of PDK1 are shown and indicated by yellow arrows B) Quantification analysis of the time course showed in panel A. PDK1 nuclear accumulation peaks at 30 minutes and decreased at control levels by 60 minutes. Scale bars: 40 μ m for panel A and 20 μ m for panel B (n=4;

ANOVA $P < 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. 0 minutes calculated by Student-Newman-Keuls post hoc test).

Figure 3. PI3K, PDK1 and phospho Akt nuclear levels and PI3K activity after ANP treatment
 A) Left panel: Representative immunoblots of PI3K, PDK1 and phospho Akt (p-Akt) detected on nuclear fractions isolated from NRCMs treated with ANP 10^{-9} M for increasing period of time (0 to 30 minutes). Lamin A/C and Rho-GDI were used as loading controls respectively for nuclear and cytosolic fractions. Right panel: Histogram plots showing quantification of immunoblots from nuclear fractions. Nuclear levels of PI3K, PDK1 and p-Akt are statistically significant at 30 minutes of ANP treatment when compared to control (White bar, control; black bar, ANP 10^{-9} M 30 minutes; $n=3$ independent experiments, $*P<0.05$, $***P<0.001$ by Student's t test). B) Left panel: Representative immunoblots of PI3K, PDK1 and phospho Akt (p-Akt) detected on cytosolic fractions isolated from NRCMs treated with ANP 10^{-9} M for increasing period of time (0 to 30 minutes). Lamin A/C and Rho-GDI were used as loading controls respectively for nuclear and cytosolic fractions. Right panel: Histogram plots showing quantification of immunoblots from cytosolic fractions. Nuclear levels of PI3K, PDK1 and p-Akt did not change after ANP treatment when compared to control (White bar, control; black bar, ANP 10^{-9} M 30 minutes; $n=3$ independent experiments, $*P<0.05$, $***P<0.001$ by Student's t test.)

Figure 4. Nuclear PI3K / PDK1 accumulation induced by stimuli such as ANP is mediated upstream of nuclear Akt accumulation

A) Immunoblot for PI3K of nuclear and cytosolic fractions isolated from NTG or Akt-nuc transgenic mice hearts. Three independent experiments are shown on the blot. Quantification revealed no statistical difference in the level of PI3K between the Akt-nuc and NTG mice both in the nuclear and in the cytosolic fractions. (data not shown). H3 and GAPDH were used as loading control for nuclear and cytosolic fraction respectively. B) Histogram quantification of the immunoblot shown in panel A. Data analysis revealed no significant differences between the groups (n=3).

Figure 5. Akt-nuc hearts display a robust response in PI3K translocation to the nucleus upon intravenous cGMP treatment

A) Confocal micrographs showing PI3K staining in Akt-nuc cardiac sections two hours after saline control (left panels) or cGMP (right panels) injection. Monochromatic panels show distributions of PI3K (green), nuclei (blue), and tropomyosin to identify myocytes (red). Scale bar represents 40 μ m. White arrows point to myocyte nuclei containing PI3K (cyan nuclei in the overlay, resulting from the merging between nuclear and anti PI3Kp110 α antibody staining.). B) Enlarged view of boxed regions from panel A. C) Representative immunoblot showing increased nuclear accumulation of PI3K in Akt-nuc mouse hearts after saline or cGMP injections. No changes were observed in cytosolic fractions (top panel). Histogram quantification of top panel with statistic is reported (n=4; * P <0.01 by Student's t test bottom panel).

Figure 6. PI3K nuclear localization parallels nuclear accumulation of phospho-Akt⁴⁷³ following myocardial infarction

A) Confocal micrographs showing PI3K (left panels) and phospho-Akt⁴⁷³ (right panels) staining, in cardiac sections from sham animals. Comparable staining was performed on cardiac sections derived from mice after three (B) or seven days (C) after MI induction. Monochromatic panels on sides of each color. Overlay show PI3K or phospho-Akt⁴⁷³ (green), nuclei (blue), and tropomyosin staining to identify myocytes (red). Inset showing an enlarged view of the white box area is present in each panel. Scale bar represents 40 μ m in the main panels and 20 in the insets. Arrows point to myocyte nuclei possessing PI3K or phospho-Akt⁴⁷³, whereas arrowheads point to non-myocyte cells possessing nuclear localized PI3K or phospho-

Akt⁴⁷³. D) Graph showing the percentage of green pixels (PI3K or phospho-Akt⁴⁷³) colocalizing with nuclear stain. (n=3; ANOVA $P<0.05$; * $p<0.001$ vs sham, \$ $p<0.001$ vs 2 day MI, # $p<0.001$ vs 4 day MI calculated by Student-Newman-Keuls post hoc test).