Ontogeny of phosphoinositide 3-kinase signaling in developing heart: effect of acute β-adrenergic stimulation

Yi-Tang Tseng, Naohiro Yano, Adam Rojan, Joan P. Stabila, Bethany G. McGonnigal, Vlad Ianus, Rajan Wadhawan, and James F. Padbury

Department of Pediatrics, Women and Infants Hospital of Rhode Island, Brown Medical School, Providence, Rhode Island

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Ontogeny of phosphoinositide 3-kinase signaling in developing heart: effect of acute β-adrenergic stimulation. Am J Physiol Heart Circ Physiol 289: H1834–H1842, 2005. First published July 8, 2005; doi:10.1152/ajpheart.00435.2005.—Signaling pathways underlie cardiomyocyte growth from hyperplasia in fetal/newborn to hypertrophy in postnatal/adult hearts are not well understood. We have shown that β-adrenergic receptor (β-AR)-mediated regulation of neonatal cardiomyocyte proliferation involves p70 ribosomal protein S6 kinase (p70S6K). Here we examined the ontogeny of phosphoinositide 3-kinase (PI3K)/p70S6K signaling pathway in rat hearts and investigated the influence of β-AR on this pathway during development. Cardiac PI3K and p70S6K1 activities were high in the embryonic day 20 fetus, decreased gradually postnatally, and were low in the adult. In contrast, p70S6K2 was barely detectable. Phosphorylation of p70S6K1, Akt, and phosphoinositide-dependent protein kinase 1 were markedly increased in late gestation and early postnatal life but not in adult hearts. Phosphatase and tensin homolog on chromosome 10 (PTEN), a negative regulator of PI3K, was highly expressed in adult hearts but only at low levels and mostly in the phosphorylated (inactivated) form in the fetus. β-AR stimulation resulted in increased cardiac p70S6K1 activity only in animals ≥2 wk old, whereas Akt level was increased in all developmental stages tested. These increases were accompanied by increased Bcl-2 associated death promoter (Ser136) phosphorylation without changes in PTEN level. Thus there is globally high input of cardiac PI3K signaling during the fetal-neonatal transition period. Inactivation of PTEN may in part contribute to the high activity of PI3K signaling, which coincides with the period of high cardiomyocyte proliferation. β-AR stimulation activates cardiac p70S6K1 and Akt in postnatal animals and may activate cardiac survival signals. These data provide further evidence for the importance of β-AR and PI3K signaling in the regulation of cardiac growth during development.

Akt, isoproterenol, phosphoinositide-dependent protein kinase 1, proliferation, phosphatase and tensin homolog on chromosome 10

Multiple extracellular signals are responsible for triggering cardiac growth, including mechanical stretch and autocrine, paracrine, and endocrine factors. Among the signaling pathways associated with these are growth factors, G protein-coupled receptor (GPCR), calmodulin/calcineurin, Ras, and MAPK, to name a few (see Ref. 33 for detailed review). Each of these factors plays important roles in regulation of growth and development of the heart. The β-adrenergic receptors (β-AR) are members of the seven-transmembrane GPCR family (13). The β-AR are known to mediate the effects of catecholamines, e.g., activation of the cardiovascular system and regulation of energy metabolism. Recently, β-AR have been recognized to play important roles in the regulation of cell growth and differentiation (55). The role of β-AR in cardiac development, however, is less clear. We have demonstrated (48) that β-AR are involved in regulation of neonatal cardiomyocyte proliferation and that this mitogenic control is mediated in large part via the p70 ribosomal protein S6 kinase (p70S6K) pathway. Thus p70S6K and its upstream kinases such as Akt and phosphoinositide-dependent protein kinase (PKD)1 may play important roles in regulation of cardiomyocyte growth, and their expression may be tightly linked to cardiac development. These proteins are critical components of the phosphoinositide 3-kinase (PI3K) signaling pathway. Akt and PKD1 are signaling proteins with pleckstrin homology domains that are recruited and activated after stimulation by hormones or growth factors. Activation of these signaling proteins affects functions of downstream targets such as p70S6K, Myc, and cyclin D, leading to regulation of a wide spectrum of cell responses including proliferation, growth, movement, and survival (12, 50). The ontogeny of this important signaling pathway in the heart has not been fully characterized. There are two subtypes of p70S6K, type 1 (p70S6K1) and type 2 (p70S6K2) (40). In this study, we examined the ontogeny of cardiac PI3K signaling in rats by measuring kinase activities of PI3K and p70S6K1/2 with immunoprecipitation in vitro kinase assay. Levels of Akt, PKD1, and the tumor suppressor phosphatase and tensin homolog on chromosome 10 (PTEN), a negative regulator of PI3K signaling, were also investigated. Alteration of sympathetic input often results in significant changes in cardiac growth. Given the roles that β-AR play (via PI3K signaling) in regulation of neonatal cardiomyocyte proliferation, it is necessary to extend the scope of study to the postnatal animal. We examined the effects of acute β-AR stimulation on the ontogeny of PI3K signaling in
vivo. The results indicate a developmentally dependent expression of the PI3K signaling pathway and suggest important roles exerted by β-AR during cardiac development.

MATERIALS AND METHODS

Reagents and animals. All chemical reagents and antibodies were purchased from Sigma and Cell Signaling Technology, respectively, unless otherwise specified. The investigation conforms with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society. The study was reviewed in advance by the Institutional Advisory Committee for the Care and Use of Animals. Timed pregnant Sprague-Dawley rats (Harlan Laboratories) were housed individually in cages with free access to water and food. At the desired developmental stages, fetal and postnatal hearts were obtained by anesthetizing rats with pentobarbital (0.5 g/kg body wt ip), snap-frozen in liquid N2, and stored at −80°C. Four embryonic hearts [embryonic day (E)19–E21] at each time point were pooled together for preparing tissue lysate. Similarly, at least two hearts were pooled together at each postnatal time point [postnatal day (P)1, P2, P3, P7, 2 wk, 3 wk, and adult]. Two-month-old male rats (~250 g) were used for adult studies. Different sets of animals were used for assaying PI3K and p70S6K1/2 activities and for Western blot analysis. For internal controls, adult rats were injected with saline or insulin (2.5 μg/g body wt ip) for 15 min before livers were collected and processed. Acute β-AR stimulation studies were carried out on rats at several developmental stages (P3, P7, 2 wk, and adult). Rats were injected with isoproterenol (Iso, 1.25 mg/kg sc) for 1 h, and hearts were obtained as described above.

In vitro kinase assays. Cardiac tissue lysates were prepared from frozen whole hearts as described previously (48). Protein concentrations were determined with the bicinchoninic acid assay (45). Equal level of input protein was confirmed by resolving part of the cardiac tissue lysates in 10% SDS-PAGE gel and visualized by Coomassie blue staining. PI3K activity was determined as described by other investigators (47). Cardiac tissue lysates (0.3 mg) were immunoprecipitated with anti-phosphotyrosine antibody (Upstate Biotechnology). A-4-Phosphatidylinositol (Avanti Polar Lipids) was used as the lipid substrate (2 μg/reaction). The immunoprecipitated lysates were washed and incubated with lipid at room temperature for 5 min. An ATP mixture containing [γ-32P]ATP (3,000 Ci/mmol, 10 μCi/ml), ATP (0.5 mM), MgCl2 (100 mM), and HEPES (55 mM, pH 7.0) was added and incubated for 10 min. In some experiments, wortmannin (10 nM) was added and assayed similarly as the negative control. The reaction was stopped by adding 80 μl of 1 N HCl. After MeOH-CHCl3 (1:1) extraction, the organic phase was spotted onto TLC plates. The results were analyzed by phosphorimaging (Bio-Rad).

For p70S6K assay, cardiac tissue lysates (0.5 mg) were immunoprecipitated with antibodies against p70S6K1 or p70S6K2 (Santa Cruz Biotechnology) or a rabbit anti-goat IgG. Kinase activity was determined by measuring phosphorylation of a specific substrate (AKRRRLSSLRA) with an S6 kinase assay kit (Upstate Biotechnology). The immunoprecipitated beads were incubated with [γ-32P]ATP-ATP-Mg2+ mix at 30°C for 90 min. A sample including 10 μl of 88% formic acid without the reaction cocktails was included as a background control. Reactions were terminated by adding 10 μl of 88% formic acid. After centrifugation the phosphorylated substrate was transferred onto Whatman P81 ion exchange cellulose chromatography paper circles, washed in 0.75% phosphoric acid, and then counted in a scintillation counter.

Western blotting. Protein levels were measured by Western blotting as previously described (48), using phospho-specific antibodies against p70S6K1 (Thr421/Ser424), Akt (Ser473, Thr308), PDK1 (Ser241), and PTEN (Ser380) and antibodies against total p70S6K1, Akt, PDK1, and PTEN (1:1,000). To assess cell apoptosis, antibodies against phospho-Bcl-2-associated death promoter (BAD) (Ser136) and total BAD were used. The results were visualized by chemilumi-

RESULTS

Ontogeny of cardiac PI3K activity. We used a monoclonal anti-phosphotyrosine antibody (4G10) to immunoprecipitate the heart lysates, thus identifying the tyrosine-phosphorylated isoform of PI3K. There are at least four different p110 (α, β, δ, and γ) catalytic subunits of PI3K in mammalian cells. Among these, the α-, β-, and γ-isomers have been shown to express in the heart (16). PI3Kα plays a critical role(s) in the development of pressure-overloaded failing heart. Overexpression of a catalytically inactive mutant of PI3Kα (PI3Kα inactive) in the mouse results in disruption of PI3Kα recruitment to agonist-activated β-AR, which prevents receptor downregulation and ameliorates development of heart failure with pressure overload (35a). We chose to assay tyrosine-phosphorylated PI3K for several reasons. First, PI3Kα is normally activated by receptor tyrosine kinase pathways and is probably the most important PI3K isoform in the regulation of cardiomyocyte growth. Targeted deletion of p110α results in a profound proliferative defect and embryonic lethality (6). In contrast, PI3Kα-null mice are healthy and fertile, with normal cardiac function and Akt phosphorylation (16). Transgenic mice that overexpress PI3Kα inactive have normal basal cardiac function (35a). This may be the best evidence that the PI3Kα isoform is not critical for neonatal cardiac development. Second, although PI3Kα can be activated by the Gβγ subunits of GPCR, this activation has only been observed in hematopoietic cells (39). The effect of Gβγ subunits of GPCR is not restricted to PI3Kα only. More evidence has indicated that PI3Kα can also be activated by the Gβγ subunits of GPCR (28). In elegant experiments using a series of transgenic mice with alterations in either p110α or p110γ, it has been shown that PI3Kα acts as a negative regulator of cardiac contractility, whereas p110α is responsible for regulation of cardiomyocyte growth (16). Third, the expression of PI3Kα is largely restricted to leukocytes and may play more roles in immunity (53). These studies suggest that the PI3Kα isoform performs more important roles in the regulation of cardiac growth and is more pertinent to our studies. Using the 4G10 antibody in our studies allowed us to focus on PI3Kα without measuring the contribution from other PI3K isoforms.

The activity of cardiac PI3K during late fetal, postnatal, and adult stages of development is shown in Fig. 1. The activity was low in E19 hearts, rose dramatically to the highest level in E20 hearts, and gradually decreased postnatally, reaching its lowest activity in adult hearts. A sudden dramatic change in the activity of a signaling molecule is not uncommon. For example, a recent study on cardiac p38 activity showed a “rhythmic” pattern of cardiac p38 activity during fetal and neonatal periods. Cardiac p38 activity is low in E12 rats, rises gradually afterward to peak activities in E14–E16 rats, and then decreases gradually to low levels (comparable to that of E12) in...
Ontogeny of cardiac PI3K activity. Cardiac tissue lysates were immunoprecipitated with an anti-phosphotyrosine antibody and subjected to in vitro lipid kinase assay using phosphatidylinositol 3-phosphate (PIP), the phosphorylated end product, is indicated by an arrow. The results were confirmed in 2 additional experiments. Equal protein loading (Coomassie blue stain) is shown (bottom).

E19 rats. This is followed by a dramatic sudden rise in E20 rats (19). As a negative control, wortmannin, a PI3K inhibitor, was included in the reaction mixture. Addition of wortmannin completely abolished the PI3K activity in the E19 heart (Fig. 1). Figure 1, bottom, shows an equal level of input protein for each of the developmental stages.

Ontogeny of cardiac p70S6K1 and p70S6K2 activities. Peak cardiac p70S6K1 activity was seen in E20–P2 animals (Fig. 2A). The activity decreased to low levels in adult hearts. We used adult liver as an internal control because adult liver p70S6K1 activity is highly responsive to insulin treatment (9). Liver samples prepared from adult rats treated with saline or insulin were used as the negative and positive controls, respectively. The high p70S6K1 activity seen from E20 (7.51 ± 0.06 pmol·min⁻¹·mg⁻¹) to P2 (6.95 ± 0.27 pmol·min⁻¹·mg⁻¹) hearts was comparable to that of insulin-treated adult liver (8.62 ± 0.34 pmol·min⁻¹·mg⁻¹). Hence, there is a high basal p70S6K1 activity in the developing heart. p70S6K2 activity, on the contrary, was not detected in fetal and newborn hearts and at only low levels (0.0283 ± 0.0093 pmol·min⁻¹·mg⁻¹) in adult hearts (Fig. 2B). In adult hearts the activities of both p70S6K1 and PI3K were significantly lower than in developing hearts. This is in contrast to liver, in which the higher basal p70S6K1 activity, although lower than that of adult heart as shown here, is seen in adults (9). This suggests that the ontogeny and regulation of PI3K and p70S6K1 are tissue specific.

Phosphorylation of cardiac PI3K, Akt, and PDK1 during development. Concerted, sequential phosphorylation events of p70S6K at multiple sites are critical for kinase activity. Among these sites, Thr389 in the hydrophobic motif of the COOH-terminal tail has been shown to increase stability but...

Phosphorylation of the intermediate kinase Akt at Thr308 and Ser473 is required for Akt activation (1). Phosphorylation of Akt at Thr308 and Ser473 was readily demonstrable starting in E20 hearts, with higher degrees of phosphorylation seen in P3 and P7 hearts (Fig. 4A). The proportion of phosphorylated protein remained significantly elevated in P7 hearts and was greatly decreased in adult hearts. Phosphorylation of PDK1 at Ser241 is critical for its activation (14). We detected highly phosphorylated PDK1 at Ser241 in E20, P3, and P7 hearts (Fig. 4B). As seen for Akt, phosphorylation of PDK1 was significantly reduced in adult hearts.

Phosphorylation of cardiac PTEN during development. PTEN is the major negative regulator of the PI3K/Akt/p70S6K signaling pathway (54). Phosphorylation of PTEN at the COOH-terminal domain is essential (21, 36, 38). There was substantial phosphorylation of p70S6K1 at Thr421/Ser424 at late gestation (E20), and the phosphorylation remained high at newborn stages (P3, P7) (Fig. 3). The phosphorylation decreased to low levels in adult heart. These results were confirmed with other phospho-specific antibodies against other critical sites such as Thr389 (not shown).
reduce activity of the phosphatase (51). We demonstrated that PTEN was highly phosphorylated at the COOH-terminal Ser380 residue, and thus inactive, in fetal and newborn hearts but not in adult hearts (Fig. 5). In contrast, total PTEN (the active form) level in adult hearts was significantly higher than that in fetal and newborn hearts. Thus a high level of PTEN, mostly in the active (nonphosphorylated) state, may in part contribute to the low PI3K signaling in adult heart. These results confirm that cardiac PI3K signaling is highly active during late gestation and newborn stages but significantly reduced in adult rats.

Effects of acute β-AR stimulation on levels of cardiac p70S6K1, Akt, and PTEN during postnatal developmental stages. Repeated agonist administration can cause desensitization of β-AR signaling in rat heart depending on the developmental stage of the animal (44). The dosing regimen for acute ISO treatment (1.25 mg/kg sc) was chosen carefully so that receptor desensitization did not occur. The same dosing regimen has been used by us and other investigators. For example, Auman et al. (3) showed that the same dosing regimen for ISO activates basal adenyl cyclase activities, measured 1–4 h after treatment, in neonatal and adult hearts. There is no homologous or heterologous desensitization of β-AR-mediated responses in neonatal heart and only a slight but not significant increase in homologous desensitization 1 h after ISO injection in adult hearts.

Levels of cardiac phospho-specific p70S6K1, Akt, and PTEN were assessed in newborn (P3, P7), postnatal (2 wk old), and adult rats with or without acute β-AR stimulation. In saline-treated rats, p70S6K1 phosphorylation (Thr421/Ser424) was high in P3–P7, low in 2-wk-old, and lowest in adult hearts (Fig. 6). Acute β-AR stimulation significantly increased phosphorylation of p70S6K1 only in 2-wk-old and adult rats. Phosphorylation of p70S6K1 in younger animals (P3, P7), which was already high, was not further increased by stimulation of β-AR.

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As shown above, adult hearts had a low degree of Akt phosphorylation (Ser473). In control hearts, although the phosphorylation of p70S6K1 was reduced significantly starting in 2-wk-old animals, the decline of Akt phosphorylation in postnatal animals was more gradual. Also different from p70S6K1, Akt phosphorylation was significantly increased by activation of \( \beta \)-H9252-AR at every developmental stage tested (Fig. 7). In contrast, the levels of PTEN were not changed by activation of \( \beta \)-H9252-AR (Fig. 8). These results demonstrate that \( \beta \)-H9252-AR stimulation results in direct increases in the levels of p70S6K1 and Akt in \( \beta \)-H11350-2-wk-old postnatal rats without the alteration of PTEN levels.

Effects of acute \( \beta \)-AR stimulation on cardiac p70S6K1 activity and BAD phosphorylation in postnatal rats. Because the levels of cardiac phospho-specific p70S6K1 were increased by \( \beta \)-AR stimulation in 2-wk-old animals, we wanted to confirm that p70S6K1 activity was increased similarly in these animals. As expected, acute \( \beta \)-AR stimulation caused an over fourfold increase (5.5 ± 1.1 vs. control 1.3 ± 0.1 pmol·min\(^{-1}·mg\)^{-1}) in cardiac p70S6K1 activity (Fig. 9A).

BAD is a proapoptotic member of the Bcl-2 family. Nonphosphorylated BAD heterodimerizes with Bcl-xL to promote cell death, whereas phosphorylated BAD is sequestered in the cytosol, which precludes its binding to Bcl-xL, and leads to enhanced cell survival (57). To assess whether acute \( \beta \)-AR stimulation results in changes in cell survival in postnatal rats, heart tissue lysates from 2-wk-old and adult rats treated with either saline or Iso were subjected to Western blot using antibodies against phospho-specific BAD (Ser136) and total BAD. The results indicate that acute stimulation of \( \beta \)-AR induces a significant increase in BAD phosphorylation in both 2-wk-old and adult rats (Fig. 9B). These results suggest that acute \( \beta \)-AR stimulation results in increases in cardiac p70S6K1 activity and may activate cardiac survival signals in postnatal rats.

DISCUSSION

This is the first report on the ontogeny of cardiac PI3K signaling and the effects of acute \( \beta \)-AR stimulation on PI3K...
ontogeny of cardiac PI3K signaling and β-AR stimulation

Fig. 8. Effects of acute β-AR stimulation on the expression of PTEN during cardiac development. Rats at several postnatal ages (P3, P7, and adult) were injected with either saline (C) or isoproterenol (I) for 1 h before death. Cardiac tissue lysates were examined by Western blot analysis for PTEN expression using a phospho-specific antibody against Ser380 and an antibody against total PTEN. Bar graph shows the results of densitometric measurement of PTEN normalized with phospho-PTEN from 3 separate experiments.

Fig. 9. Effects of acute β-AR stimulation on p70S6K1 activity and Bcl-2-associated death promoter (BAD) expression in postnatal heart. Rats were injected with either saline (C) or isoproterenol (I, Iso, 1.25 mg/kg sc) for 1 h before death. A: cardiac p70S6K1 activity in 2-wk-old rats was determined in vitro kinase assay using anti-p70S6K1 antibody. Shown are results from 3 separate experiments. *P  0.01 vs. control. B: phosphorylation of BAD was determined using a phospho-specific (Ser136) antibody and an antibody against total BAD. Bar graph shows the results of densitometric measurement of phospho-BAD normalized with total BAD from 3 separate experiments. *P < 0.01 vs. control of the same developmental age.

signaling in vivo. Our overall goals were first to examine the levels and activity of PI3K signaling during cardiac development and second to investigate the effects of acute β-AR stimulation on cardiac PI3K signaling in postnatal animals. The ontogeny of cardiac PI3K signaling appears to be highly regulated, with consistently elevated activity during late gestation and early newborn stages and significantly lower activity in postnatal and adult hearts. We show that cardiac PTEN phosphatase level is inversely correlated with the ontogeny of PI3K signaling. Acute β-AR stimulation in vivo can only activate PI3K signaling when the signaling has already waned in postnatal (2 wk old and beyond) hearts. We further demonstrate that short-term activation of β-AR also results in BAD phosphorylation, suggesting an increase in cell survival in postnatal hearts.

It has been shown that cardiac PI3K activity and level remain constant in postnatal animals from P1 to P20 (26). The cardiac PI3K activities in these animals, however, are significantly higher than those of adults. Although we also show a significantly reduced PI3K signaling in adult heart, our data demonstrate a temporal peaking of the cardiac PI3K signaling from late gestation to early postnatal stages only. The aforementioned study was performed with an antibody against the p85 regulatory subunit of PI3K, whereas in our study a phosphotyrosine antibody was used. Hence, it is possible that the level of p85 regulatory subunit is constant (26) while the actual, total PI3K activity decreases gradually in the postnatal heart (Fig. 1).

PDK1 has been shown to activate both p70S6K and Akt (2). The phosphoactive forms of these kinases were highly expressed in the heart, with peak levels seen in late gestation (PDK1) or early postnatal (Akt, p70S6K1) stages and the lowest activity seen in adults. This developmental pattern is similar to that of other cell cycle regulators such as cyclins and cyclin-dependent kinases (CDK) during cardiac development (10). The elevated levels and activity of PI3K signaling may play critical roles in cardiomyocyte proliferation during these periods of development. Although the link between PI3K/Akt/p70S6K activation and hypertrophy is well documented (16, 42), recent studies also suggest a role of PI3K signaling in promoting cell proliferation. For example, it has been shown that Akt regulates cell proliferation by phosphorylating the CDK inhibitor (CDKI) p27kip1, causing retention of p27kip1 in the cytoplasm and thus preventing it from inducing cell cycle G1 arrest (32, 41, 52). Moreover, Akt activation has been shown to cause hypertrophy as well as hyperplasia in other cell types (5, 49). We have demonstrated (48) that β-AR-mediated regulation of neonatal cardiomyocyte proliferation is at least partly related to PI3K signaling. Levels and activities of the PI3K signaling and positive regulators of the cell cycle are significantly downregulated in adult heart, which coincides with the loss of proliferative capacity in cardiomyocytes. Furthermore, inhibition of PI3K has been shown to induce G1 cell cycle arrest and to inhibit cell proliferation via involvement of cyclin D1, CDK, and CDC25A (24). Hence, the shift of cardiomyocyte growth from hyperplasia to hypertrophy may involve, among other factors, both the PI3K signaling pathway and cell cycle regulators such as cyclin, CDK, and CDKI.

PTEN is a tumor suppressor gene, and deletion of the gene has been found in many tumor types (31, 46). PTEN phospho-
tase functions as a negative regulator of PI3K signaling by catalyzing dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate, effectively reversing the effect of PI3K (34). Both PI3K and PTEN have been shown to be critical in regulation of cardiac growth (16, 42). In transgenic mice, cardiac-specific expression of a constitutively active form of PI3K results in larger hearts and dominant-negative PI3K results in smaller hearts (42). Cardiac-specific inactivation of PTEN results in hyper trophy and reduction in contractility (16). The 50-amino acid COOH-terminal tail of PTEN is an inhibitory domain, and phosphorylation of PTEN at three residues (Ser380, Thr382, and Thr383) of the COOH-terminal tail has been shown to increase stability but reduce activity of the phosphatase (51). We demonstrated highly phosphorylated PTEN (Ser380) in late gestation and early postnatal hearts but not in adult hearts. The total PTEN levels, however, were markedly elevated in adult hearts. Thus PTEN is expressed at a low level and exists mostly in the inactive state in fetal and newborn hearts. In contrast, PTEN is expressed at a high level and exists in the active form in adult hearts. This suggests that low PTEN phosphatase activity helps maintain a high PI3K/p70S6K signaling environment during the proliferative periods of cardiac development. By the same token, high PTEN expression may be the main contributor to the low activity of cardiac PI3K signaling in the adult heart.

The role of sympathetic input in the regulation of cardiac growth is of great interest. Hypertrophic cardiac growth is associated with congestive heart failure, in part due to overstimulation by catecholamines. It is widely accepted that alteration of sympathetic input results in significant changes in cardiac growth. For example, subhypertensive doses of norepinephrine and sustained activation of β-AR from infusion of ISO induce significant cardiac hypertrophy (22, 29). Transgenic mice with cardiac-specific overexpression of the human β1-AR have gene dose-dependent cardiac hypertrophy and suffer from histopathological damage and myocardial dysfunction (7). Moreover, β-AR antagonists given to pregnant dams result in offspring with significant impairment of cardiac growth and a delay in cardiac cellular development (27). These studies demonstrate the important roles that β-AR play in regulation of cardiac development. Our laboratory is interested in the roles of β-AR in regulation of cardiomyocyte growth during fetal-postnatal transition. We previously demonstrated (48) that β-AR blockade significantly reduces cardiac proliferation index and p70S6K1 activity in neonatal rats. These studies were conducted when the heart was in the hyperplastic growth mode. Although we have not demonstrated this in the fetal heart, these studies nonetheless suggest that the β-AR input plays an important role in cardiomyocyte growth regulation in a PI3K signaling-dependent manner.

We show here that β-AR stimulation does not increase PI3K signaling in neonatal hearts. This can be explained by the fact that PI3K signaling is already at its maximal expression. Activity at this developmental stage hence could not be stimulated further. This is supported by the high cardiac p70S6K1 activity in rats from E20 to P2, when it matches the level detected in insulin-stimulated liver (Fig. 2). On the contrary, β-AR stimulation significantly increases the levels of Akt and levels and activity of p70S6K1 in postnatal and adult hearts, in which the PI3K signaling has diminished to low levels. The levels of PTEN were, however, not affected by the same treatment, suggesting a direct action of acute β-AR stimulation on PI3K signaling in postnatal hearts. During development, β-AR stimulation-induced receptor desensitization or down-regulation is not present until 2 wk of age (3, 44). The absence of β-AR desensitization in early development may be physiologically critical, as β-AR signaling is required for maintaining proper homeostasis and growth of the heart. In a sense, this is reminiscent of the absence of the increase in PI3K signaling by β-AR stimulation in the developing heart as shown in our study. Between P6 and P15 there is a change in the expression of G protein subunits, when Gα1 (52 kDa) and Gα2 decline significantly but Gα1 (45 kDa) and Gα1,2 remain unchanged (56). Although the levels of these G proteins can be modulated by chronic β-AR stimulation, the effect of acute β-AR stimulation has not been investigated. Hence, it remains to be seen whether the effects of acute β-AR stimulation on activation of PI3K signaling in 2-wk-old animals is linked to the switch over in the expression of G protein subunits.

BAD phosphorylation can be induced by multiple effectors, including Akt, p70S6K, PKA, and the Ras-MAPK (Rsk2) signaling pathway (8), although different sites are phosphorylated by these effectors. These sites are Ser112, Ser116, and Ser155, and they may be equally important regarding their relevance to apoptosis as point mutations of all three sites abolish BAD’s protective effect against cell death (18). Akt and Rsk2 exhibit preference for BAD phosphorylation at Ser136 and Ser112, respectively (8). We demonstrated increases in cardiac p70S6K1 activity and in Akt and BAD (Ser136) phosphorylation after acute β-AR stimulation in postnatal animals (2 wk old). This suggests an increase in cardiomyocyte survival by acute β-AR stimulation. Although we did not measure ERK1/2 MAPK, we previously showed (48) that in neonatal heart blockade of β-AR reduces p70S6K1 activity without affecting the level of ERK1/2. Moreover, there is a close similarity in phenotype between mice with point mutations of all three BAD phosphorylation sites and mice with Akt1 knocked out (18). It has been shown that activation of Akt promotes cell survival by phosphorylating BAD at Ser136 (17). Cardiac-specific overexpression of Akt not only protects against myocyte apoptosis but also reduces infarct size and improves cardiac function after ischemia-reperfusion injury (23, 35). However, although we clearly observed a significant increase in BAD phosphorylation by acute β-AR stimulation, we did not confirm cardiomyocyte survival with other assays such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, flow cytometry, caspase activation and DNA fragmentation. Our study nonetheless is the first to demonstrate an acute treatment regimen of β-AR agonist to induce survival signaling in vivo.

Induction of cardiomyocyte apoptosis by in vitro or in vivo β-AR stimulation has been demonstrated (43, 58). Although the detailed mechanism underlying β-AR-mediated cardiomyocyte apoptosis is not well understood, recent evidence suggests that in adult rat ventricular myocytes (ARVM) this may involve reactive oxygen species and JNK-dependent activation of the mitochondria death pathways (15). It has been further shown that β1-AR stimulation induces cardiomyocyte apoptosis, whereas β2-AR stimulation activates predominantly a G1/G2-γ-PI3K-Akt-mediated survival signaling in adult mouse cardiomyocytes in vitro (15a, 58). A majority of the studies demonstrating a β1-AR-mediated increase in apoptosis were...
performed in vitro with ARVM (15a, 43). These studies required treatment of cells with β-AR agonists for prolonged time periods, usually between 12 and 24 h. Whether these prolonged treatments induce receptor downregulation, hence contrasting with our findings, was not examined. Our studies were performed under an acute Iso treatment regimen (1 h). Our observation that β-AR stimulation activates Akt/p70S6K1 and may increase survival (increased BAD phosphorylation) is consistent with those studies demonstrating a β2-AR-mediated decrease in apoptosis (15a, 58). The effect of short-term β-AR stimulation on cardiac survival signaling, however, needs to be confirmed, and the effect of β-AR stimulation on cardiac proliferation in vivo during development should be investigated further. Akt can be activated by several cardioprotective signaling molecules, such as IGF-I (11). Our study, however, did not address the question of how β-AR stimulation results in activation of Akt and downstream signals. One possibility is the activation of epidermal growth factor receptor/Pi3K via a Gβγ/Src-mediated signaling pathway (20).

In summary, the activity of the Pi3K signaling pathway during cardiac development is highly regulated. There is a globally high input of the cardiac Pi3K signaling during late gestation and early postnatal but not postnatal and adult stages. The high activities of these proteins occur when cardiomyocytes are undergoing hyperplastic growth. In postnatal animals, acute β-AR stimulation results in activation of cardiac Pi3K signaling and may increase cell survival. These data provide further evidence for the importance of β-AR and Pi3K signaling pathways in regulation of cardiac growth during development.

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