ORIGINAL ARTICLE

PI3K Phosphorylation Is Linked to Improved Electrical Excitability in an *In Vitro* Engineered Heart Tissue Disease Model System

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Myocardial infarction, a prevalent cardiovascular disease, is associated with cardiomyocyte cell death, and eventually heart failure. Cardiac tissue engineering has provided hopes for alternative treatment options, and high-fidelity tissue models for drug discovery. The signal transduction mechanisms relayed in response to mechanoelectrical (physical) stimulation or biochemical stimulation (hormones, cytokines, or drugs) in engineered heart tissues (EHTs) are poorly understood. In this study, an EHT model was used to elucidate the signaling mechanisms involved when insulin was applied in the presence of electrical stimulation, a stimulus that mimics functional heart tissue environment *in vitro*. EHTs were insulin treated, electrically stimulated, or applied in combination (insulin and electrical stimulation). Electrical excitability parameters (excitation threshold and maximum capture rate) were measured. Protein kinase B (AKT) and phosphatidylinositol-3-kinase (PI3K) phosphorylation revealed that insulin and electrical stimulation relayed electrical excitability through two separate signaling cascades, while there was a negative crosstalk between sustained activation of AKT and PI3K.

Introduction

CARDIAC TISSUE ENGINEERING aims to create replacement tissue for regenerative medicine applications, and *in vitro* model systems for drug discovery.¹⁻⁴ Significant advances have been made with generating engineered heart tissue (EHT), including varieties of cell sources from neonatal mouse and rat cells, as well as human cardiomyocytes derived from human pluripotent stem cells.^{5,6} Most notably, it has been emphasized that reproducing some aspects of the native cardiac tissue environments through mechanical stretch, electrical stimulation, or culture medium perfusion is critical for successful long-term cultivation of EHTs.⁷⁻⁹

However, despite these technological advances, very few studies have focused on understanding the molecular pathways that govern the assembly of cells in EHTs and their function.¹⁰ Uncovering these mechanisms will enable us to expand the scope of drug targets that can be tested using human *in vitro* tissue arrays, as well as to improve culti-

vation methods and to develop strategies for promoting survival of the tissue upon transplantation.

A key component in the native myocardium is physical stimulation such as mechanical load and electrical stimulation, which modulates cell function and tissue morphology. External pacing of the tissue by electrical stimulation has been shown to mimic the excitation-contraction coupling that occurs in the healthy myocardium.⁷⁻⁹ The mechanical contraction initiated by the external pacing aligns the cardiomyocytes (CMs) to the electrical field, resulting in the development of a mature phenotype found in the native heart.¹¹

We aimed to assess how the electrical properties of the myocardium are affected upon treatment with insulin, a commonly utilized protein in cardiac disease patients and tissue-engineered constructs, by investigating the kinase phosphorylation upon insulin addition in an *in vitro* EHT cultivation environment, while electrical field stimulation was applied. Since the phosphatidylinositol-3-kinase (PI3K) pathway is involved in cytoskeletal rearrangement, and previous monolayer studies showed that PI3K blockers

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abolished the beneficial effects of electrical stimulation on neonatal rat cardiomyocyte morphology,¹² we hypothesized that PI3K pathway could be involved in the transduction of electrical stimulation signals during three-dimensional (3D) EHT cultivation.

Insulin has been studied as a potential cardiac drug target which when administered in myocardial infarction (MI) patients resulted in an increased survival after MI especially in diabetic patients.¹³ In cardiac tissue engineering, an optimized culture media containing insulin increased the force of contraction as measured by twitch tension in EHTs.¹⁴ However, it was not clear if this was a direct effect of insulin on electrical excitability parameters, or whether some other downstream pathways were activated. IGF-1, a growth factor known to have similar downstream signaling pathways as insulin,¹⁵ and an indistinguishable effect on glycogenesis,¹⁶ improved the electrical excitability properties of EHTs in previous studies,¹⁷ by lowering the excitation threshold (ET) and increasing contractile amplitude. Since IGF-1 has been shown to stimulate cell growth and survival by phosphorylating protein kinase B (AKT),¹⁸ and prevent apoptosis through phosphorylation of PI3K pathway,¹⁹ we hypothesized that the insulin treatment of EHTs might also activate PI3K and AKT pathways. We also hypothesized that coapplication of insulin and electrical field stimulation might have a synergistic effect on electrical excitability parameters, tissue morphology, and downstream pathway activation.

In this study, we also assessed how insulin affected the electrical properties in a two-dimensional (2D) model. Previous *in vitro* studies have found that there were substantial differences in contraction ability and cytoskeleton rearrangement when cardiac cells were grown in a 3D context compared to 2D.²⁰ Understanding the physiological and molecular differences of insulin treatment under the presence of electrical stimulation in 2D and 3D environment will extend our knowledge on how we could utilize EHT to benefit in the discovery of new pharmacological agents to improve cardiac performance. In accordance with our previous data,¹² 2D cardiomyocyte cultivation indicated that there were no differences in either electrical properties or kinase activation in the control, insulin, and combined conditions; ET, the minimum voltage for EHTs to contract at 1 beat per minute worsened in the electrical stimulation only condition in comparison to the control. In our 3D model, which we previously have shown can mimic the physiological conditions of the native myocardium,²¹ we observed 3D EHTs to be more sensitive to the applied conditions. 3D EHT experiments indicated that no synergistic effects were observed upon simultaneous insulin treatment and electrical stimulation; insulin administration sustained AKT phosphorylation, while electrical field stimulation acted through PI3K. In EHTs with coapplication of insulin and electrical field stimulation, only AKT phosphorylation was sustained; inhibition of AKT phosphorylation using API-2 in the combined condition was associated with PI3K phosphorylation.

Materials and Methods

Cell isolation

Neonatal (1–2 days old) Sprague-Dawley rats (Charles River) were euthanized by decapitation according to the procedure approved by the Institute's Committee on Animal

Care (ethics approval protocol 20009788). The atria were removed and the hearts were exercised, quartered, and enriched. CMs were isolated by an overnight trypsin (6120 U/mL; Sigma-Aldrich) incubation in Hank's balanced salt solution (HBSS) at 4°C followed by a series of collagenase digestion (220 U/mL; Worthington Biochemical) in HBSS at 37°C for 8 min each as previously described. The supernatants from five collagenase tissue digestions were collected and centrifuged at 750 rpm (94 g) for 5 min, resuspended in a culture medium (Dulbecco's modified Eagle's medium [DMEM], 4.5 g/L glucose; Gibco), supplemented with 10% certified fetal bovine serum (FBS; Gibco), 100 U/mL penicillin-streptomycin and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid buffer (HEPES; Gibco), and preplated onto T75 flasks for 1 h. The nonadhered cells from T75 flasks were collected and counted for live cells after Trypan blue staining to determine the cell density.

Construct preparation and assessment of electrical excitability parameters

Dry Gelfoam collagen sponge scaffolds (Gelfoam; Delasco) (1 cm × 1.5 cm × 300 μm) were prewetted with culture media, and the enriched CMs (1.0 million cells per scaffold) were seeded using Matrigel (10 μL per scaffold; Becton-Dickinson) (Fig. 1A). After 30 min gelation period, all constructs were cultivated for 2 days in the high glucose DMEM media, a standard cell seeding procedure. Subsequently, the constructs were transferred to their respective culture media (Insulin [I9278; Sigma] 20 μU/mL; LY294002 [1130; Tocris] 10 μM; 740 Y-P [1983; Tocris] 10 μg/mL; API-2 [2151; Tocris] 0.1 μM), and maintained in this media until the end of 8 day cultivation. For the 2D experiments, acrylic sheets were laser cut to create a template (0.8 × 0.7 cm) to seed cardiomyocytes. After overnight incubation with fibronectin and gelatin mixture, CMs (0.1 million cells per construct) were seeded. Using our best protocol for neonatal rat cardiomyocytes cultivation, found beneficial in a previous study,⁷ half of the EHTs and 2D constructs were stimulated using an external stimulator (Grass 88×; AstroMed) at 1 Hz, repeating biphasic pulse with duration at 0.001 s, and voltage (V) at 6 V/cm during the last 5 days in a custom-made electrical stimulation chamber (Fig. 1B). On day 8 of cultivation, electrical excitability parameters (ET, maximum capture rate [MCR]), which have been correlated with improvements in structure and function of a human induced pluripotent cardiac tissue in a biowire platform,²² were recorded (Fig. 1C, D). As illustrated in Figure 1C, to analyze ET values, external voltage was increased until the tissue began to contract synchronously at 1 Hz. In Figure 1C, ET was 7 V/cm since it was the minimum required for the EHT to contract at 1 Hz. As shown in Figure 1D, to analyze MCR, the electrical stimulation rate was increased until the EHT no longer responded. In Figure 1D, MCR value was 2 Hz since it was the highest rate the EHT responded to.

Cell viability assessment using flow cytometry analysis

On day 8 of cultivation, EHTs were dissociated with 0.1% collagenase IV for 30 min at 37°C with an intermittent gentle pipetting until there were no clumped cells. Cells were spun down in phosphate buffered saline, and

FIG. 1. Experimental setup from neonatal cardiomyocytes to engineered heart tissues (EHTs) with insulin and/or electrical stimulation. **(A)** Rat neonatal cardiomyocytes were seeded onto the 300 μ m collagen scaffold and then cultivated in a custom-made electrical chamber; experimental time line for the cultivation of EHTs and application of insulin or electrical stimulation. **(B)** Custom-made electrical stimulation chamber. **(C)** Excitation threshold (ET) is measured by increasing the voltage until EHTs synchronously contract. **(D)** Maximum capture rate (MCR) is measured by increasing electrical stimulation rate until the EHT can respond to. Color images available online at www.liebertpub.com/tea

propidium iodide staining was performed according to the manufacturer's protocol (Molecular Probes) and analyzed using flow cytometry (BD FACScanto).

Quantitative polymerase chain reaction

RNA was isolated by an RNeasy Mini Kit (Qiagen). Reverse transcriptase reaction was carried out by using SuperScript III (Invitrogen) with 0.5 μ g of total RNA. Quantitative polymerase chain reactions (qPCRs) were performed at 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 60°C for 1 min, 72°C for 15 s followed by dissociation step using 7900HT Fast Real-Time PCR system (Applied Biosystems). The following primers were used in qPCR: Myosin Heavy Chain α (MHC α)—forward 5'-TGATGACTCCGAGGAGCTTT-3', reverse 5'-TGACACAGACCCTTGAGCAG-3'; Myosin Heavy Chain β (MHC β)—forward 5'-CCTCGCAATATCAAGGGA-3', reverse 5'-TACAGGTGCATCAGCTCCAG-3'; PI3K—forward 5'-AGCCACAGGTGAAAATACGG-3', reverse 5'-TTTTCTTTCCGCAACAGCTT-3'; natriuretic peptide precursor A (nppa)—forward 5'-ggggtaggattgacaggat-3', reverse 5'-ctccaggagggtattcacca-3'; β -actin—forward 5'-TAAAGACCTCTATGCCAACAC-3', reverse 5'-GATAGAGCCACCAATCCAC-3'.

Western blotting

After cultivating the EHTs for 8 days, each tissue was placed into a tube containing 350 μ L of lysis buffer (Protein Prep) with 3.5 μ L of phosphatase inhibitor 1 and 2. These tubes were then incubated overnight at 4°C on a shaker. The Micro BCA Protein Assay Kit (Thermo Scientific) was used to measure the protein concentration in each of these samples. Cell lysate was resolved by SDS-PAGE (Any kD Mini-PROTEAN Precast Gel, #456-9034; BioRad) and then transferred onto polyvinylidene difluoride (PVDF) Immun-Blot membrane (10 $\times$ 5 cm; BioRad). The membrane was blocked with 2.5% milk for 1 h and then washed with Tris-buffered saline and Tween 20 (TBS-T) 1% for 5 min at room temperature. Subsequently, all

primary antibodies were incubated overnight at 4°C while secondary antibodies were left for 1 h at room temperature before development. After each antibody incubation step, the membrane was washed five times for 5 min with 2.5% milk, and one time with TBS-T 1%. ECL Plus Western Blotting Detection System (Amersham GE Healthcare) was used for development. Primary antibodies used for western blotting were as follows: rabbit anti-phospho-AKT (Ser473, 1:2000; Cell Signaling), rabbit anti-phospho-PI3K (Tyr458, 1:1000; Cell Signaling), rabbit anti-GAPDH (1:10,000; Cell Signaling), mouse anti-total AKT (1:2000; Cell Signaling), rabbit anti-total PI3K (1:1000; Cell Signaling). Secondary rabbit (1:5000; R&D systems), and mouse antibody (1:5000; R&D systems) were used as appropriate. Western Hyperfilm was bought from Amersham GE Healthcare.

All quantifications for western blots were done using ImageJ. As shown in figures, phosphorylation of AKT or PI3K was both normalized to GAPDH and either Total AKT or Total PI3K. Normalizations to GAPDH can be found in the supplementary documents while normalizations to Total AKT or Total PI3K will be shown and described in the text of the results.

Statistical analyses

Results are expressed as mean $\pm$ SEM. Statistical significance was assessed using Tukey's test followed by one-way analysis of variance or Dunn's test for more than three groups (SigmaStat), or *t*-test in only two groups compared and denoted with * when the *p*-value for at least three independent experiments was less than 0.05.

Results

Insulin and electrical stimulation enhanced functional properties of the engineered heart tissues, but did not significantly impact tissue morphology and gene expression

EHTs that were cultivated in the presence of insulin, electrical stimulation, or combination condition (Fig. 2A) had

improved electrical excitability parameters [lower ET (Fig. 2B) and higher MCR (Fig. 2C)], when compared to EHTs cultivated in the control, stimulation-free condition. This was in contrast to the 2D studies (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea), where there was no difference in electrical properties, with the exception of worsened ET in the electrical stimulation condition in comparison to the control condition (Supplementary Fig. S1A, B), and the monolayers exhibited a markedly lower MCR compared to the EHTs. Even though the insulin only or electrical stimulation only condition was sufficient to improve electrical excitability parameters, the combination condition did not exhibit an additive benefit in 3D on electrical excitability properties in contrast to our original hypothesis. Therefore, we performed cellular and molecular analyses to investigate possible mechanisms that each stimulus or combination of both exhibit on the EHTs after 8 days of cultivation.

Histological analysis indicated no appreciable differences among different cultivation conditions, and histologically all the EHTs showed similar tissue morphology (data not shown). Investigation of total cellular protein (Fig. 3A) and % of cell viability, using propidium iodide staining followed by flow cytometry analysis, showed no differences among all the treatment conditions (Fig. 3B and Supplementary Figs. S2 and S3). Since the electrical excitability properties in the combination condition did not improve compared to the insulin or electrical stimulation only conditions, we examined gene expression profiles related to the contractile proteins. Figure 4A shows that insulin, electrical stimulation, or combination condition decreased the ratio of β -MHC to α -MHC when compared to the control condition,

indicating maturation in the rodent cardiomyocytes settings with application of stimuli such as insulin or electrical stimulation. The magnitude of the ratio was the same in the three conditions; the combination condition did not further decrease the ratio. While MHC is known to be involved in force production, PI3K has been previously reported to be related to cytoskeletal rearrangement in cardiomyocytes.¹² In this study, the presence of electrical stimulation showed a

FIG. 3. Cellular composition of EHTs. (A) Total protein amount in each condition was quantified ($n=5$) with the BCA Protein Assay Kit (ThermoScientific). Total protein amount nonsignificant across conditions ($p>0.05$). (B) % of propidium iodide+ cells from digested EHTs as a measure of cell viability. No difference in cell viability across conditions were observed ($p>0.05$) ($n=5$).

FIG. 4. Similarity among gene expression profiles among conditions. The mRNA expression was normalized to β -actin and compared to the control condition. **(A)** β -MHC to α -MHC ratio. **(B)** PI3K. (* $p < 0.05$, $n = 4-7$). PI3K, phosphatidylinositol-3-kinase.

tendency to increase PI3K mRNA; this, however, did not reach the statistical significance (Fig. 4B, $p = 0.1$ [electrical stimulation only], $p = 0.12$ [combination of insulin and electrical stimulation] compared to the control).

Insulin phosphorylates AKT while electrical stimulation phosphorylates PI3K

Signaling mechanisms were investigated through western blots after finding no conclusive data that would explain the trends in electrical excitability parameters in the insulin, electrical stimulation, and combination condition. Western blot analysis on the phosphorylation of AKT and PI3K were performed after cultivation for 8 days. In Figure 5A and Supplementary Fig. S4, we show that the addition of insulin sustained phosphorylated AKT compared to other conditions.

Next, we examined PI3K phosphorylation. Figure 5B and Supplementary Fig. S5 indicate that electrical stimulation sustained robust phosphorylation of PI3K on day 8. Contrary to the electrical stimulation condition, treating EHTs with insulin for 8 days showed no sustained PI3K phosphorylation. While electrically stimulated EHTs exhibited an increase in phospho-PI3K, the combination condition did not sustain phosphorylation of PI3K. Since cell viability, known to be involved in PI3K/AKT phosphorylation, was not significantly different among the treatment conditions (Fig. 3B and Supplementary Figs S2 and S3), we suggest phosphorylation of kinases was correlated with increased electrical excitability properties.

Taken together, our data suggest that EHTs cultivated in the presence of insulin had enhanced AKT phosphorylation, while those cultivated with electrical stimulation exhibited enhanced phosphorylation of PI3K after 8 days of cultivation. In tissues that were cultivated in the combination condition, phosphorylation of AKT was seen alongside improved electrical excitability properties; however, the extent of AKT phosphorylation was reduced when compared to the insulin only condition. In addition, no appreciable PI3K phosphorylation was observed in the combination condition (Fig. 5B).

Presence of PI3K inhibitor LY294002 blocked phosphorylation of PI3K in electrically stimulated EHTs

To verify whether electrical stimulation improves electrical excitability parameters through sustained PI3K phosphorylation, PI3K inhibitor, LY294002 was added in the presence or absence of electrical stimulation (Supplementary Fig. S6A). The addition of LY294002 demonstrates that this molecule blocks the phosphorylation of PI3K, which is the pathway involved in improving the electrical excitability parameters (* $p < 0.05$) (Fig. 6C and Supplementary Fig. S6). Electrical excitability parameters mirrored the pattern of PI3K phosphorylation, and

FIG. 5. AKT is phosphorylated in insulin-treated EHTs while PI3K is phosphorylated upon electrical stimulation. **(A)** Western blot analysis to detect phospho-AKT, total AKT, and GAPDH. Western blot for phospho-AKT/Total AKT (* $p < 0.05$, $n = 7$). All blots were quantified using ImageJ. **(B)** Western blot analysis to detect phospho-PI3K, total PI3K, and GAPDH. Western blot for phospho-PI3K/Total PI3K. (* $p < 0.05$, $n = 5-6$). AKT, protein kinase B.

FIG. 6. Presence of PI3K inhibitor LY294002 aggravates electrical excitability parameters in stimulated EHTs. **(A)** ET, **(B)** MCR, **(C)** Western blot analysis to detect phospho-PI3K, total PI3K, and GAPDH. Western blot for phospho-PI3K; phospho-PI3K/Total PI3K (* $p < 0.05$, $n = 5$).

specifically the addition of LY294002 in the electrically stimulated condition increased ET to the control level. However, the addition of LY294002 to the control condition did not exhibit appreciable effects on ET (Fig. 6A). MCR had an inverse trend compared to ET, as expected. Specifically, the highest MCR was observed in the electrically stimulated EHTs, while the addition of the PI3K inhibitor significantly decreased MCR levels to the values consistent with the control condition (Fig. 6B). The PI3K inhibitor study confirmed that electrical field stimulation during culture resulted in PI3K phosphorylation. The presence of the inhibitor in electrically stimulated EHTs abolished phosphorylation of PI3K, diminishing electrical excitability properties on day 8.

Presence of PI3K activator (740 Y-P) in EHTs with insulin does not sustain phosphorylation of PI3K

Since the combination condition was shown to phosphorylate AKT, we hypothesized that activating both PI3K and

AKT would lead to better electrical excitability properties. Therefore, we used 740 Y-P, a PI3K only activator (Supplementary Fig. S7), to pharmacologically activate PI3K phosphorylation simultaneously with AKT phosphorylation (Supplementary Fig. S8A). Sustained phosphorylation of PI3K after 8 days of cultivation by the addition of 740 Y-P to the control condition was observed as expected (Fig. 7C and Supplementary Fig. S8). However, the addition of 740 Y-P to the combination condition (electrical stimulation + insulin) did not sustain phosphorylation of PI3K (Fig. 7C and Supplementary Fig. S8). Similarly, in the insulin only condition, presence of 740 Y-P did not induce sustained phosphorylation of PI3K. These data suggest that there is either an interfering mechanism between PI3K and AKT activation in EHTs in the presence of insulin, or that on day 8, changing the timing when both stimuli are initiated (e.g., from day 2 to 3 or later) would enable phosphorylation of both kinases.

In terms of electrical excitability parameters, although the addition of 740 Y-P was able to improve these parameters in

FIG. 7. Presence of a PI3K activator, 740 Y-P, increased phosphorylation of PI3K in EHTs without insulin or electrical stimulation. **(A)** ET **(B)** MCR. **(C)** Western blot analysis to detect phospho-PI3K, total PI3K, and GAPDH. Western blot for phospho-PI3K/Total PI3K. (* $p < 0.05$, $n = 5$).

our control condition, presence of 740 Y-P did not further induce better electrical excitability properties in conditions with insulin or electrical stimulation with respect to their counterpart without 740 Y-P additions (Fig. 7A, B).

In summary, insulin sustained phosphorylation of AKT while electrical stimulation sustained phosphorylation of PI3K on day 8. A PI3K inhibitor LY294002 study verified that electrical stimulation specifically activated and sustained PI3K signaling, which was responsible for improved electrical excitability properties. On the other hand, the use of 740 Y-P, a PI3K activator, showed that phosphorylation of PI3K in the control condition could improve electrical excitability parameters while additional effects were not seen in conditions that had insulin sustained phosphorylated AKT.

Presence of AKT inhibitor (API-2) in EHTs with insulin sustains phosphorylation of PI3K

Since the combination condition had only sustained phosphorylation of AKT on day 8, we hypothesized that an interfering mechanism may exist between insulin-activated AKT and electrical stimulation-activated PI3K. To investigate this further, we treated our combined condition with API-2, an AKT inhibitor to verify whether an interfering mechanism between AKT and PI3K exists. In EHTs that were treated with API-2, we found that their electrical properties did not change compared to their control counterparts (the combined condition had similar electrical properties to the combined condition treated with API-2). (Supplementary Fig. S9A, B). Western blots revealed (Supplementary Fig. S9C) ($n=3-4$) that insulin-mediated AKT phosphorylation was successfully inhibited upon treatment of API-2; inhibition of AKT phosphorylation resulted in the upregulation of phospho-PI3K (Supplementary Fig. S9C). When the insulin only patches were treated with API-2, an AKT inhibitor, we hypothesized that electrical properties

would be comparable to those in the control condition; this, however, was not the case. Western blots revealed that insulin only EHTs treated with API-2 had an inverse relationship between sustained AKT and PI3K; decreased phosphorylation of AKT was seen alongside an increase in sustained phosphorylated PI3K; this resulted in EHTs with insulin and API-2 treatment having better electrical properties than the control condition. In the combined condition post-API-2 treatment, we found that when there was a decrease in AKT phosphorylation, there was an increase in sustained phosphorylated PI3K. Since both phosphorylation of AKT and PI3K had an inverse relationship, this confirmed our hypothesis that insulin-activated AKT inhibited electrical stimulation-mediated phosphorylation of PI3K.

Discussion

Molecular analyses of *in vitro*-generated tissues have been rare, and given the increasing importance of micro-tissue and -organ engineering in drug development and disease modeling, is an important new area of focus. The goal of this study was to develop a model that will enable us to elucidate the signaling pathways relayed to electrical excitability properties when our chemical and electrical stimulation was applied in synchrony. It is important to acknowledge that PI3K and AKT are two out of the many phosphoproteomes that could have been assessed, and that phosphorylation of kinases could have occurred at any point throughout the course of the experiment.

Figure 8 shows our proposed model of the signaling pathways activated under the presence of insulin, electrical stimulation, and the combination condition, respectively. First, insulin treatment sustained phosphorylation of AKT, resulting in better electrical excitability properties; phosphorylated PI3K was not sustained on day 8 by insulin treatment only (Fig. 8A). Although insulin has been shown

FIG. 8. Proposed kinase phosphorylation model in EHT under insulin and electrical field stimulation. (A) Insulin sustains phosphorylation of AKT to induce better electrical excitability properties; (B) Electrical stimulation sustains phosphorylation of PI3K to improve electrical excitability properties. When phosphorylation of PI3K is inhibited by LY294002, improvements in electrical stimulation-induced effects are lost. (C) Despite presence of 740 Y-P, a PI3K activator, in the presence of insulin, phosphorylation of PI3K was not sustained. Color images available online at www.liebertpub.com/tea

to activate the PI3K/AKT signaling cascade, all documented results are from short-term treatment (less than 60 min) of insulin or IGF in *in vitro* cell culture settings.²³

In the *in vivo* transgenic mouse models, overexpression of AKT is associated with both physiological and pathological responses. It has been shown that cardiac-specific constitutively active AKT transgenic mice improved myocardial contractility²⁴ by increased expression of SERCA2a, due to a physiological hypertrophic response,²⁵ and decreased infarct size after ischemia–reperfusion injury²⁶; however, chronic AKT activation also induces contractile dysfunction in the aged mice,²⁷ but beneficial effects in lipopolysaccharide-induced cardiac dysfunction.²⁸ Results from cardiac-specific inducible AKT1 transgenic mice demonstrated that transient activation of AKT (2 weeks) caused reversible hypertrophy, but 6 weeks of activation induced impaired contractility,²⁹ and that short-term AKT activation improved contractile function in pressure overload- or Adriamycin-induced heart failure.³⁰ Chronic activation of AKT pathway was evident in the failing hearts from patients,³¹ and an *in vitro* study on simulated ischemia–reperfusion has shown that during simulated ischemia, there was an absence of AKT activation, while ischemia–reperfusion treatment induced AKT activation.³²

One of the novel aspects of this study is that we were able to recapitulate the integration and interference of two kinase signaling pathways, chronic AKT and PI3K in the engineering heart tissue, which was shown in the myocardium of transgenic mice. In our study, the chronic PI3K was shown to be activated through electrical stimulation especially in the 3D model, which supports the hypothesis that not only biochemical signals, but also mechanoelectrical signals have a significant effect on cardiomyocyte physiology. As stated previously, overexpression of AKT in transgenic mice had shown to induce cardiac hypertrophy and sudden death,²⁶ while also impairing cardiac contractile function.³³ Another study in which two transgenic mice that overexpressed PI3K and AKT were bred, found that the ratio of heart size was significantly lower in mice that had been produced by crossbreeding a chronic AKT transgenic mouse with a knockdown PI3K mouse.³⁴

There is very little known about the combined effects of chronic AKT and PI3K activation in the myocardium, but it is well known that overexpression of AKT or PI3K can lead to a hypertrophic myocardium with poor contractile properties. In our study, long-term treatment of insulin (AKT activator) and electrical stimulation (PI3K activator) sustained phosphorylation of AKT, suggesting the possibility of a negative feedback loop that inhibits the long-term activation of PI3K; our AKT inhibitor study helps confirm this. When insulin-dependent AKT inhibited sustained phosphorylation of PI3K, this negative feedback loop eliminated chronic activation of both AKT and PI3K in EHTs, which otherwise may have been detrimental to its contractile and cellular properties.

The correlation between sustained phosphorylation of AKT (8 days) by insulin treatment and enhanced electrical excitability parameters without changing cell survival in our study might represent an excellent intermediate tissue-engineered model for further studies on hypertrophy and insulin resistance, given that the role of the insulin receptor and its autophosphorylation (Tyr 1150/1551)^{35,36} in this con-

text is established. In addition to using this tissue-engineered model to study pathological conditions, if the excitation coupling can be mimicked and its underlying mechanisms are understood, the uses for this model could be significantly increased. Our data show that cardiomyocytes respond differently in terms of electrical excitability parameters (ET, MCR) when they are insulin treated in a 2D system versus 3D EHT system. Specifically, 3D EHT system was more sensitive to the externally applied stimuli, insulin, and electrical field stimulation compared to the 2D system.

Application of electrical stimulation to EHTs sustained phosphorylation of PI3K on day 8, also resulting in better electrical excitability properties; this effect was completely blocked by PI3K inhibitor, LY294002 (Fig. 8B). Unlike insulin treatment, electrically stimulated EHTs sustained phosphorylated PI3K without sustained phosphorylation of AKT.

PI3K (class I), an upstream kinase of AKT, is a heterodimer consisting of two subunits, the p85 regulatory and p110 catalytic subunits. Upon activation, p85 mediates translocation of p110, bringing p110 in contact with their lipid substrates in the cell membrane. Upon translocation of p110, downstream kinases like AKT are activated.³⁷ There are multiple different stimuli that have been reported to activate PI3K for catalytic activity; however, it seems that PI3K has kinase-independent functions. Results have shown that focal adhesion kinase binds to p85, and this interaction was increased by mechanical stretch.³⁸ Treatment of LY294002, a PI3K inhibitor, to the ventricles of the heart decreased intracellular Ca^{2+} and contraction frequency, thus demonstrating that PI3K is implicated in excitation–contraction coupling through activation of L-type Ca^{2+} channels.³⁹ However, in *in vivo* mouse models, overexpressing dominant negative PI3K (p110 α) did not cause dysfunction in contractility.⁴⁰ Interestingly, PI3K (p110 γ) deletion in the hearts increased cardiac contractility.⁴¹

Contrary to our hypothesis that both PI3K and AKT would be sustained in the combination condition, only sustained AKT was evident on day 8 (Fig. 8C); addition of a PI3K activator, 740 Y-P, did not activate PI3K independently in the presence of AKT activation.

While it has been reported that treatment of cells by insulin exerts effects through the PI3K/AKT signaling cascade, recent *in vivo* studies suggest the possibility of independent activation and feedback between PI3K and AKT. Transgenic mice with cardiac-specific expression of myristoylated AKT (chronic AKT activation) have shown negative inhibition of PI3K,⁴² indicating the possibility of a negative feedback loop from AKT to PI3K. In a mouse study on exercise adaptations, while it has been accepted that the PI3K/AKT signaling cascade^{43,44} helps with exercise adaptations in the heart, only phosphorylation of PI3K without AKT was necessary for physiological adaptations.⁴⁵

In cancer cells, inhibiting AKT released AKT-dependent feedback inhibition resulting in activation of insulin and IGF receptors, which in turn attenuated the drug's effect.⁴⁶ Also, the balance between PI3K and AKT molecules seem to be tightly regulated; overexpression of nonphosphorylated form of p85 subunit of PI3K decreases AKT phosphorylation by inhibiting p110 kinase activity, while on the other hand, phosphorylated form of p85 induced AKT phosphorylation.⁴⁷ Previously, the MAPK/ERK1 gene expression was

observed to be upregulated when epithelial cells were electrically stimulated to enhance wound healing⁴⁸; future studies examining MAPK expression could provide insight on how closely gene and protein expressions are linked in cardiomyocytes undergoing stimulation, and how different pacing frequencies can affect protein upregulation levels. Also, it would be interesting to further investigate the effect of pacing frequency on the PI3K activation on the short-term and long-term electrical stimulation, since we have previously showed that human cells had to pace at 6 Hz possibly due to their immature developmental stage.²²

Conclusion

An EHT model that combines both biochemical and physical stimulation is required to recapitulate the milieu of a native myocardium and study convergence of signal transduction from different kinds of stimulation. In our model, when the biochemical stimulus (insulin treatment) was applied in the presence of physical stimulation (electrical pacing), sustained phosphorylation of both PI3K and AKT was not simultaneously observed on day 8 even though insulin treatment and electrical stimulation when applied independently, were able to sustain phosphorylation of AKT and PI3K, respectively.

Modulating kinase phosphorylation and their synergistic effects is useful when discovering specific combinations of both biochemical and physical stimulation to treat cardiomyocyte damage from MI. Examining with single cell resolution can also offer insight about how variability between genetically identical cells affects electrical excitability parameters, while also explaining if there is a synergistic effect coming from cell coupling of many single cells. In addition to our pharmacological experiments, treating EHTs with PI3K/AKT small interfering RNAs, alongside other biochemical stimuli can (1) firmly establish the suggested link among the kinases and, (2) investigate the interactive effects of two or more biochemical stimuli in the presence of physical stimulation.

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Disclosure Statement

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