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Impaired translocation and activation of mitochondrial Akt1 mitigated mitochondrial oxidative phosphorylation Complex V activity in diabetic myocardium

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Abstract

Insulin can translocate Akt to mitochondria in cardiac muscle. The goals of this study were to define sub-mitochondrial localization of the translocated Akt, to dissect the effects of insulin on Akt isoform translocation, and to determine the direct effect of mitochondrial Akt activation on Complex V activity in normal and diabetic myocardium. The translocated Akt sequentially localized to the mitochondrial intermembrane space, inner membrane, and matrix. To confirm Akt translocation, *in vitro* import assay showed rapid entry of Akt into mitochondria. Akt isoforms were differentially regulated by insulin stimulation, only Akt1 translocated into mitochondria. In the insulin-resistant Type 2 diabetes model, Akt1 translocation was blunted. Mitochondrial activation of Akt1 increased Complex V activity by 24% in normal myocardium *in vivo* and restored Complex V activity in diabetic myocardium. Basal mitochondrial Complex V activity was lower by 22% in the Akt1^{-/-} myocardium. Insulin-stimulated Complex V activity was not impaired in the Akt1^{-/-} myocardium, due to compensatory translocation of Akt2 to mitochondria. Akt1 is the primary isoform that relayed insulin signaling to mitochondria and modulated mitochondrial Complex V activity. Activation of mitochondrial Akt1 enhanced ATP production

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Disclosure statement
None.

and increased phosphocreatine in cardiac muscle cells. Dysregulation of this signal pathway might impair mitochondrial bioenergetics in diabetic myocardium.

1. Introduction

The complex interactions between cytosolic signaling and mitochondrial signaling and its pathophysiological implications on the regulation of bioenergetics in cardiac muscle are largely unknown. Downstream of phosphatidylinositol-3 kinase (PI3K), Akt/PKB is an important signaling step for insulin and other growth factor receptors and plays a major role in the regulation of metabolism, growth, and survival [1–3]. Previous studies on Akt had concentrated largely on cytosolic Akt and nuclear Akt. Akt is best known for its regulatory effects on metabolism. Akt activates GLUT4 translocation and glucose uptake through the AS160-Rab pathway and promotes glycolysis [4,5]. Cytosolic Akt signaling might augment ATP production by modulating hexokinase [6], and ablation of Akt drastically decreased ATP content in fibroblasts [7]. Our laboratory recently reported acute translocation of Akt into mitochondria by insulin stimulation in cardiac muscle cells [8]. Since the effect of insulin on oxidative phosphorylation (OXPHOS) Complex V was blocked by LY294002, we hypothesized that activation of mitochondrial Akt would promote Complex V activity.

There are three isoforms of Akt in mammalian cells, Akt1, Akt2 and Akt3. Akt isoforms share >80% homology and were encoded by separate genes [1,9]. All three Akt isoforms contain an N-terminal Pleckstrin homology domain, a kinase domain, and a hydrophobic regulatory domain. Akt1 and Akt2 are believed to exist in all mammalian cells, whereas Akt3 is not expressed in muscle [10]. Divergent phenotypes in Akt isoform knockout mice suggested that each Akt isoform possessed a distinct regulatory function *in vivo* [11–15]. Akt1 and Akt2 are the two major isoforms of Akt in cardiac muscle [16,17]. Whether insulin differentially regulates Akt1 and Akt2 translocation to mitochondria is not known. To gain insight into mitochondrial Akt signaling, we set out to define the sub-mitochondrial localization of Akt in cardiac muscle cells in response to insulin stimulation.

Human diabetic cardiomyopathy is accompanied by reduced myocardial phosphocreatine/ATP ratio, indicating impaired high energy phosphate-metabolism and energy deficit [18–20]. Impaired mitochondrial oxidative phosphorylation and lower ATP synthesis rates were also observed in animal models of diabetes [21–25]. In our previous study [8], insulin-induced Akt translocation to mitochondria was impaired in the myocardium of diabetic mice. The goals of this study were to define sub-mitochondrial localization of the translocated Akt, to dissect the effects of insulin on Akt1 and Akt2 translocation and activation, and to determine the direct effect of mitochondrial Akt activation on Complex V activity in normal and diabetic myocardium.

2. Materials and methods

2.1. Materials

Anti-Akt, anti-phosphoserine 473-Akt, anti-Akt1 and anti-Akt2 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-porin and anti-complex Vd were from MitoSciences (Eugene, Oregon, USA) and Life Technologies (Carlsbad, CA). Other antibodies were from Genetex (Irvine, CA). Recombinant human insulin was from Novo Nordisk (Princeton, NJ). Other chemicals were from Sigma or Fisher Scientific. Akt1 and Akt2 knockout mice were obtained from Jackson Laboratory (Bar Harbor, ME).

2.2. Experimental animals

C57BL/6 mice and Sprague–Dawley (SD) rats were from Harlan (Indianapolis, IN). Streptozotocin (STZ)-induced diabetes was achieved by injecting STZ (80 mg/kg body weight, i.p.) into SD rats (approximately 200 gram body weight), or by injecting STZ (200 mg/kg body weight, i.p.) into 10- to 12-week-old C57BL/6 mice. Insulin-resistant diabetes was induced by feeding 10- to 12-week-old C57BL/6 mice with high fat (42%) chow and 60% fructose drinking water for 6–8 weeks. Blood glucose levels were monitored by tail-vein sampling. Diabetes was verified by random blood glucose > 200 mg/dL. For acute insulin stimulation, under anesthesia insulin (1 U/kg body weight) was injected into the inferior vena cava after overnight fasting [26]. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee at University of California, Irvine.

2.3. Mitochondrial preparation

Myocardium was cut into small pieces and homogenized in a Potter-Elvehjem tissue grinder vessel with MSB buffer (mannitol 225 mM, 75 mM sucrose, 0.5% BSA and 5 mM HEPES pH 7.2) and 100 U/ml collagenase. Two mM EGTA was added after incubating the homogenates on ice for 1 min to chelate free Ca^{2+} . Differential centrifugation was carried out to collect mitochondrial pellets [8]. Mitochondrial pellets were resuspended in mitochondrial isolation buffer (MIB, 225 mM mannitol, 75 mM sucrose, 10 mM MOPS [pH 7.2], 1 mM EGTA, and 0.5% BSA) supplemented with 3 $\mu\text{g}/\text{ml}$ aprotinin, 3 $\mu\text{g}/\text{ml}$ leupeptin, 2 mM phenylmethyl-sulfonyl fluoride (PMSF), 20 mM NaF, 10 mM NaPP, and 2 mM Na_3VO_4 , or in a mitochondrial buffer (20 mM HEPES–KOH pH 7.2, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) with protease and phosphatase inhibitors.

2.4. OXPHO Complex V activity assay

Mitochondrial membranes were ruptured by freeze–thaw cycles in MIB buffer, and OXPHO Complex V activity were measured as previously described [27,28]. In brief, equal amounts of mitochondrial proteins were added to 800 μl pre-warmed distilled water and 200 μl pre-warmed reaction buffer containing 50 mM Tris–HCl (pH 8.8), 1 mM NADH, 5 mg/ml BSA, 20 mM MgCl_2 , 50 mM KCl, 2.5 mM ATP, 15 μM carbonyl cyanide *m*-chlorophenylhydrazone, 10 mM phosphoenol pyruvate, 5 μM antimycin and 4 U of lactate dehydrogenase/pyruvate kinase at 37 °C. The activity was measured by the absorbance at 340 nm for 3 min. Twelve μM oligomycin was added to the reaction mixture to determine the oligomycin-sensitive Complex V activity (between 1st 3 min reading and 2nd 3 min reading). The abundance of Complex V subunits were analyzed with Complex V Immunocapture Kit (Mitosciences) as instructed by the manufacturer.

2.5. Subfractionation of mitochondria

One hundred μg mitochondria were treated with 100 μl proteinase K (30 $\mu\text{g}/\text{ml}$, in PBS), as described in our previous study to remove non-mitochondrial protein contamination [8]. To subfractionate mitochondrial compartments, mitochondria were treated with 180 $\mu\text{g}/\text{ml}$ digitonin on ice for 20 min, and then with 400 μl of swelling buffer (10 mM KH_2PO_4 , pH 7.4, 3 $\mu\text{g}/\text{ml}$ aprotinin, 3 $\mu\text{g}/\text{ml}$ leupeptin, 2 mM PMSF, 20 mM NaF, 10 mM NaPP, and 2 mM Na_3VO_4) and incubated at 4 °C for 20 min with gentle mixing. An equal amount of shrinking buffer (10 mM KH_2PO_4 , pH 7.4, 32% sucrose, 30% glycerol, and 10 mM MgCl_2) was added and incubated at 4 °C for another 20 min. The mitochondrial suspension was centrifuged at 10,000 g for 10 min. The supernatants contained outer membrane (OM) and inner membrane space (IMS) proteins. The pellet was re-suspended in 300 μl of swelling buffer and sonicated three times at 15 W for 10 s, followed by adding 0.01% NP-40 and

incubated on ice for 10 min. The preparation was centrifuged at 145,000 *g* for 1 h with a Beckman SW 50.1 rotor. The resulting pellet represented the mitochondrial inner membrane fraction (IM).

To separate mitochondrial membranes from the matrix fractions (Mx), mitochondria were sonicated at 40 W for 15 s, incubated on ice for 1 min and sonicated for a second time. The resulting mixtures were ultracentrifuged at 150,000 *g* for 1 h. The resulting pellets represented the membrane fractions (OM, IMS, and IM) and the supernatants represented matrix fractions. During subfractionation, the specificity of each fraction was verified by immunoblotting with antibodies against specific protein markers.

2.6. In vitro import of p-Akt into mitochondria

Mitochondrial fractions were isolated from un-stimulated mouse myocardium and the cytosolic fractions were isolated from insulin-stimulated mouse myocardium for the following experiments. The cytosolic fractions, which contained activated Akt, was suspended in 80 mM KCl, 5 mM MgCl₂, 2 mM KH₂PO₄, 1 mM ATP, 4 mM NADH, 5 mM creatine phosphate and 100 µg/ml creatine phosphate kinase. The import assay was started by mixing the cytosolic fractions with un-stimulated mitochondria in a 25 °C water bath for the indicated time periods. A mitochondria free sample was included as a negative control. AVO (8 µM Antimycin, 1 µM valinomycin and 12 µM oligomycin) was used to disrupt the mitochondrial cross-membrane electrochemical gradient when indicated. At the end of the assay, mitochondria were collected by centrifugation and digested with Proteinase K to remove non-mitochondrial proteins, solubilized, and immunoblotted for the presence of p-Akt.

2.7. Immunofluorescence staining

To visualize the effect of insulin on Akt1 subcellular localization, the cardiomyocytes were fixed with 4% formaldehyde for 30 min at room temperature. After washing with PBS, cells were treated with 0.05% saponin in ddH₂O for 20 min and blocked with 10% normal sera for 30 min. The fixed cells were incubated with specific primary antibodies overnight at 4 °C, and conjugated secondary antibodies for 1 h, counter-stained with DAPI, and analyzed with Eclipse Ti fluorescence microscope (Nikon).

2.8. Overexpression of a mitochondria-targeting, constitutively active Akt1 in myocardium

Adenovirus was injected into the myocardium via a subdiaphragmatic approach [29]. SD rats were anesthetized and incisions were made at the abdomen below the diaphragm using sterile techniques. The sternum was lifted with forceps and the apex of the ventricle could be identified through the diaphragm. Forty µl of adenovirus (Ad-GFP or 1:1 mixture of Ad-mito-Akt and Ad-GFP) was injected through the diaphragm into the left ventricular wall using a 29-gauge syringe. Each animal was injected twice. After the injections, the abdomen was sutured and rats were allowed to recover. Four days after the injections, the rats were sacrificed and the ventricular apex portion was isolated and the presence of GFP fluorescence was used to guide dissection. The ventricular wall without GFP was collected as an internal control.

2.9. Transducing cardiomyocytes with mitochondria-targeting constitutive active Akt1 and mitochondria-targeting dominant negative Akt

Two recombinant adenoviruses were used in this study. The constitutive active mitochondria-targeting Akt1 (Ad-mito-Akt) and a dominant negative Akt (substitutions at K179A, T308A and S473A) with mitochondrial targeting sequence at the N-terminal was

constructed as described in our previous publications, and transduction of cardiomyocytes with adenoviral and lentiviral vectors was carried out as previously described [8,30].

2.10. Assays for ATP and mitochondrial calcium

Cultured neonatal rat cardiomyocytes were trypsinized and counted before use. For each assay, 1×10^6 cells were used. ENLITEN ATP assay kits were obtained from Promega. Cells were digested with 2.5% TCA and then neutralized with Tris-acetate (pH 7.75). The luminescence was measured using Synergy™ HT multi-detection microplate reader. A 2-second delay time after 100 μ l rLuciferase/Luciferin reagent injection and a 10-second RLU signal integration time were used according to the ENLITEN ATP assay manual. Mitochondrial calcium level was analyzed by rhod-2 AM (Molecular Probes). Cardiomyocytes were incubated with rhod-2 (1 mM) for 15 min at 37 °C and washed 3 times with PBS. The fluorescence was measured with BD LSR II Flow Cytometer (excitation at 488 nm and emission at 576 nm) and the data were analyzed with BD FACSDiVa software.

2.11. Analysis of phosphocreatine with mass spectrometry

Cells were washed with PBS, scraped, snap-frozen by liquid nitrogen, and stored at -80°C for further analysis. To extract the metabolites, the cell pellets were re-suspended in 200 μ l ddH₂O and 800 μ l HPLC-grade methanol, extraction was performed at room temperature for 24 h. The soluble metabolites were collected by centrifuging the mixtures for 5 min, the supernatants were dried with a Speedvac. The dried pellet was dissolved in 50 μ l 50% CH₃CN and 0.1% TFA. 1 μ l of the resulting extract was mixed with 2 μ l of matrices and spotted onto an Opti-TOF 384-well plate. Three replicates were spotted for a given extraction and three different extractions were carried out from a cell sample. The matrices were prepared by dissolving CHCA (10 mg/ml) or DHB (50 mg/ml) in 50% CH₃CN and 0.1% TFA. Creatine and phosphocreatine (Sigma) were used as standard references. MS analysis was performed with an AB Sciex 5800 MALDI-TOF/TOF analyzer. Prior to MS analysis, calibration in the reflector positive mode was performed with creatine and phosphocreatine and the resultant parameters were used as default calibration. Mass spectrometry analyses were acquired in the positive ion mode.

2.12. Statistical analysis

The data were presented as mean $\pm$ SEM, from the results of three to six independent experiments. The intensity of bands from blots was scanned with densitometry and digitally analyzed. Statistical significance was tested with one-way ANOVA and post hoc analysis with Newman-Keuls test when appropriate. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Reconstitution of Akt transport into mitochondria

We first determined whether the effects of insulin on Akt mitochondrial translocation required activation of Akt kinase itself. To this end, we studied the effect of a PI3K inhibitor LY294002 on insulin-induced Akt protein translocation. Insulin stimulation acutely increased the abundance of mitochondrial p-Akt, which was blunted by the PI3K inhibitor LY294002 (Fig. 1A, upper panel). However, LY294002 did not inhibit Akt protein translocation to mitochondria (Fig. 1A, lower panel). This finding suggested that Akt translocation to mitochondria did not require activation of Akt kinase itself, but may require activation of an additional signaling pathway.

Our previous experiments on Akt translocation relied on cell compartment subfractionation [8]. To confirm and reconstitute translocation of Akt upon insulin stimulation, we carried

out an *in vitro* import assay with isolated mitochondria. Mitochondria prepared from unstimulated myocardium and cytosolic fractions from insulin-stimulated myocardium were used for this assay. The cytosolic fractions, which contained insulin-stimulated Akt, were mixed with the un-stimulated mitochondria in the presence of ATP, NADH, and creatine phosphate and incubated at 25 °C for the indicated time periods. The mitochondria were harvested, washed, digested with Proteinase K to remove non-mitochondrial proteins, and analyzed with immunoblots (Fig. 1B). p-Akt could be detected in the mitochondria as early as 2 min after incubating with insulin-stimulated cytosolic proteins. The abundance of p-Akt gradually decreased in the mitochondria but remained detectable after 60 min of incubation. Collapsing the mitochondrial transmembrane proton gradient with AVO inhibited Akt import, suggesting that an adequate proton gradient is required for Akt import into mitochondria.

3.2. Sub-mitochondrial localization of the insulin-stimulated Akt

It is important to know where Akt localizes upon entrance to mitochondria. The next series of experiments analyzed the submitochondrial localization of translocated Akt after insulin stimulation. Two different approaches were used in these experiments. First, we isolated the membrane fractions from the mitochondrial preparation and separated the inner membrane (IM) fraction from the outer membrane (OM) and intermembrane space (IMS) fractions (Fig. 1C). p-Akt was detected in the OM + IMS fractions 2 min after insulin stimulation. p-Akt was not detectable in the IM fractions until after 5–15 min of insulin stimulation, which suggests sequential movement of Akt from the OM to the IM. In the second approach, intact mitochondria were disrupted by sonication and subfractionated into membrane fractions and matrix fractions (Fig. 1D). p-Akt was detected in both the membrane fractions and matrix after insulin stimulation, suggesting that Akt reached the mitochondrial matrix.

3.2.1. The acute effect of insulin stimulation on Akt isoform translocation in normal and diabetic myocardium—There are three Akt isoforms in mammalian cells but myocardium predominantly expresses two isoforms—Akt1 and Akt2 [16,17]. To investigate the effect of insulin on Akt isoform translocation to mitochondria, specific Akt1 and Akt2 antibodies were used for immunoblotting of purified mitochondria (Fig. 2). Interestingly, only Akt1 could be translocated to mitochondria after insulin stimulation in normal myocardium (Fig. 2), indicating that Akt1 is the major isoform that translocated into mitochondria in cardiac muscle. To determine whether the effect of insulin on Akt isoform translocation was altered in diabetic myocardium, we studied isoform translocation in two different models of diabetes. A Type 1 Diabetes (insulin deficient) model was created by streptozotocin (STZ) injection, and a Type 2 Diabetes (insulin resistant) model was induced by high fat/high fructose (HFF) diet as we previously described [8]. Fasting blood glucose was elevated in both models (control: 143.9 ± 7.9 mg/dL; STZ: 506.1 ± 28.7 mg/dL; HFF: 192.1 ± 17.1 mg/dL). In the STZ model, insulin receptor signaling is augmented because of compensatory upregulation of the insulin receptor; in the HFF model insulin receptor signaling is diminished because of insulin resistance [8]. As shown in Figs. 2B and D, insulin stimulation exaggerated Akt1 translocation in the STZ diabetes model (after 5 days of diabetes), whereas the effect of insulin was decreased in the HFF model. Basal levels of mitochondrial Akt1 were higher in the STZ model and lower in the HFF model. Although there was no statistically significant mitochondrial translocation of Akt2 in the control mice, insulin induced Akt2 translocation in the STZ model. These results suggest that although insulin did not increase Akt2 translocation under normal condition, amplified insulin receptor signaling, such as the insulin-deprived STZ myocardium, might lead to translocation of Akt2.

In order to visualize and analyze the distribution of p-Akt1 in cardiomyocytes upon insulin stimulation, cardiomyocytes were serum-deprived overnight and stimulated with insulin (Fig. 3). The results showed that a major fraction of p-Akt1 localized to mitochondria after insulin stimulation (Fig. 3A). These data suggested that mitochondrion is a key target of Akt1 signaling upon insulin stimulation. To explore whether p-Akt1 co-localized to Hsp90, insulin-stimulated cardiomyocytes were stained for p-Akt1 and Hsp90, and the results showed minimal p-Akt1 co-localization with Hsp90 (Fig. 3B).

3.2.2. Insulin-stimulated Akt isoform translocation in Akt1^{-/-} and Akt2^{-/-} mice—In order to further dissect the acute effect of insulin on Akt isoform translocation to mitochondria, and understand the role that mitochondrial Akt1 plays in regulating Complex V activity, we investigated the insulin effect on Akt isoform translocation and Complex V activity in the Akt1^{-/-} and Akt2^{-/-} mice (A1KO and A2KO). A1KO featured a smaller body size (23.8 ± 0.6 g vs. 29 ± 0.8 g wildtype) and A2KO had a slightly elevated fasting glucose level (144 ± 8 mg/dL vs. 122 ± 7 mg/dL wildtype). As shown in Fig. 4A, insulin increased Akt1 translocation in both wildtype and A2KO myocardium. Akt1 was not detected in A1KO myocardium. Akt2 was not translocated upon insulin stimulation in the wildtype mice. However, there was significant compensatory translocation of Akt2 in the A1KO mice upon insulin stimulation (Fig. 4B). Myocardial mitochondria were isolated for analysis of Complex V activity (Fig. 4C). At basal, Complex V activity was significantly lower in the A1KO mice, but not in A2KO mice. Insulin-stimulated Complex V activity was not different between the wildtype and A1KO, suggesting that the compensatory Akt2 translocation served as an alternative pathway and mediated the insulin effect in A1KO myocardium. The P:O ratio, which measures the efficiency of respiration, was also lower in the A1KO myocardium (Fig. 4D). These data indicated that Akt1 is the major isoform that mediated insulin receptor signaling to mitochondria and modulated respiration, but Akt2 served as an alternative signaling pathway when there was no Akt1.

3.2.3. Restoration of mitochondrial Complex V activity by transducing diabetic myocardium with a constitutively active Akt1—In order to establish the causal relationship between mitochondrial Akt1 activation and Complex V activity, we transduced rat myocardium with a mitochondria-targeting constitutively active Akt1 (Ad-mito-Akt) (Fig. 5). A control virus, Ad-GFP, was co-injected into the myocardium. The overexpressed mito-Akt localized to mitochondria, and did not alter cytosolic Akt activity [30]. The presence of GFP fluorescence was used to guide and isolate the myocardium expressing the transgene. Ad-GFP-injected myocardium showed uniform expression of GFP (Fig. 5B). The mitochondria were isolated for western blot and Complex V assay. Mitochondrial Akt protein was increased in the myocardium transduced with Ad-mito-Akt but the abundance of mitochondrial Complex V subunits was not changed (Figs. 5C and D). Complex V activity increased by approximately 24% in the non-diabetic control myocardium injected with Ad-mito-Akt (Fig. 5E). In the STZ-DM rats, Complex V activity was decreased in the Ad-GFP-injected myocardium as expected, whereas in the Ad-mito-Akt-injected myocardium Complex V activity was increased to a level similar to the normal controls. Therefore, activation of Akt1 in mitochondria increased Complex V activity, and augmentation of mitochondrial Akt1 activation in diabetic myocardium restored Complex V activity.

To further analyze the effect of mitochondrial Akt1 on ATP, cardiomyocytes were transduced with control vector, Ad-mito-Akt, or a mitochondria-targeting dominant negative Akt (Fig. 5F). ATP content was higher in the cells transduced with Ad-mito-Akt and lowered in the cells transduced with the dominant negative Akt, indicating that mitochondrial Akt1 signaling modulated mitochondrial oxidative phosphorylation in cardiac muscle. To further study mitochondrial Akt1 effect on the quickly obtainable reserve of high energy phosphates, the content of phosphocreatine was determined with mass spectrometry.

As expected, phosphocreatine levels were significantly increased in cardiac muscle cells overexpressing mito-Akt and phosphocreatine:ATP ratio was accordingly increased (Fig. 5G). Since mitochondrial calcium signaling may negatively modulate mitochondrial function and respiration, we also measured mitochondrial calcium levels (Fig. 5H). Mitochondrial calcium level was increased in the cells transduced with dominant negative Akt, suggesting involvement of mitochondrial calcium signaling in this process.

4. Discussion

In this article we provided evidence that mitochondrion is a major subcellular location where Akt1 translocation/activation occurred. Myocardial energy production must respond to the dynamic changes of the cardiovascular system and meets its physiological demands [31]. The results presented in this study indicate that insulin plays a role in the rapid modulation of myocardial respiration through mitochondrial Akt1. Insulin stimulation has been implicated as an important process which enhanced ATP production [32,33]. Insulin-mediated Akt1 translocation and activation in mitochondria represents a signal mechanism linking extracellular fuel supply and hormonal response to coordinate bioenergetic supply and demand in cardiac muscle.

Mitochondria are composed of different compartments to carry out specific functions. The outer membrane contains various channels that allow entrance of large proteins with specific mitochondria-targeting sequence on their N-terminals [33]. The intermembrane space is between the outer and inner membranes, and contains several pro-apoptotic proteins, such as cytochrome *c* and AIF [34,35]. The inner membrane contains oxidative phosphorylation complexes and accessory proteins for protein and metabolite transport. The inner membranes enclose the matrix, which holds a reservoir to store proteins and chemicals needed for biochemical reactions and mitochondrial DNA and RNA maintenance and synthesis. Some oxidative phosphorylation complexes interface the intermembrane space, inner membrane and matrix. For example, the F1 portion of Complex V (ATP synthase) anchors on the inner membrane and protrudes into the matrix [36]. The presence of Akt1 in the intermembrane space, inner membrane and matrix implies that it co-localized in the same mitochondrial compartment with critical mitochondrial proteins regulating oxidative phosphorylation.

Akt1 and Akt2 are the major isoforms in skeletal muscle and cardiac muscle. Akt1^{-/-} mice exhibited intra-uterine growth retardation and Akt2^{-/-} mice were hyperglycemic and insulin resistant [11–13]. In cardiac muscle, Akt1 regulates cardiac growth and contractile function [37]. While Akt1^{-/-} mice had smaller hearts, the heart size in Akt2^{-/-} mice was not affected [38,39]. Akt1^{-/-} mice did not exhibit a myocardial hypertrophic phenotype when subjected to physiological hypertrophic stimulation [37,38], and overexpression of Akt1 in myocardium protected against cardiac injuries [37]. Akt1 and Akt2 might partially compensate for the deficiency of each other in these knockout models [40,41]. While Akt1 and Akt2 were assigned distinctive physiological roles, they might serve as an alternative signaling pathway for each other in the isoform-specific knockout mice.

At least some of the divergent functions of Akt1 and Akt2 could be explained by their different subcellular localizations. In adipocytes, insulin stimulation leads to a greater accumulation of Akt2 in plasma membranes to facilitate glucose transport [41]. Enhancing the Akt1 plasma membrane targeting through a mutated Akt1 PH domain overcame Akt isoform specificity effects on glucose transport [42], which supports the concept that Akt isoforms differ from each other by their subcellular spatial distribution. Our data revealed that Akt1 and Akt2 were differentially translocated to the mitochondria by insulin stimulation under normal conditions. Although some p-Akt1 remained in the cytosol after

insulin stimulation and did not translocate, the majority of p-Akt1 were translocated to mitochondria and the nucleus. In the isoform-specific knockout model, Akt1 and Akt2 may potentially serve as alternative signaling pathways for each other. Insulin-stimulated Complex V activity in A1KO mice did not differ from the wildtype mice, suggesting a compensatory Akt2 translocation to mitochondria. In the more extreme case of insulin deficiency such as the STZ-DM model, the insulin-stimulated Akt1 translocation was enhanced due to a compensatory increase in insulin receptor signaling. Interestingly, insulin stimulation also caused a moderate translocation of Akt2 to mitochondria in STZ-DM. Whether Akt2 could be an alternative pathway in severe insulin deficiency will require further confirmation.

Creatine kinase and phosphocreatine are key elements of the energy reserve system in heart muscle, and heart failure is accompanied by lower phosphocreatine levels as ATP levels fall. Low phosphocreatine to ATP ratio has been proposed as a predictor of mortality in heart failure patients [43]. Our results suggested that impaired mitochondrial Akt1 action contributed to a reduced high-energy phosphate reserve in diabetic cardiomyopathy. However, a limitation of our study is that we had measured cardiac muscle high energy phosphates in the asystole cardiac muscle cells, therefore we could not assess the changes of high energy phosphates during heartbeat cycle.

There are multiple potential phosphorylation sites on oxidative phosphorylation complexes [44,45]. Whether these phosphorylation sites can be regulated by intracellular signaling largely remains unknown. However, some studies have begun to shed light on potential roles of hormone signaling. For example, cytochrome *c* oxidase can be phosphorylated by PKA at serines 115/116 of subunit I, threonine 52 of subunit IV, and serine 40 of subunit Vb [45]. Translocation of Shp-2, Src family kinases and PKC δ to the mitochondria also have been described in the literature [44,46,47], but their substrates in mitochondria have not been identified. We have searched and failed to identify any Akt substrate consensus motif RXXRX(S/T) in the subunits of Complex V. Manning et al. reported that approximately 25% out of over 100 reported Akt substrates in the literature did not contain the consensus motif [4]. Whether Akt1 modulates Complex V activity through direct or indirect signaling interactions is not yet known.

Impaired energy homeostasis contributes to the development of diabetic cardiomyopathy [48]. Understanding the mechanisms of insulin receptor signaling to mitochondria may help develop new strategies to prevent or treat diabetic cardiomyopathy in insulin-deficient and insulin-resistant patients.

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Fig. 1.

Insulin-stimulated Akt translocation and activation in cardiac mitochondria. **A:** Insulin-stimulated Akt translocation to mitochondria was phosphorylation-independent. Mice were overnight-fasted and anesthetized. When indicated, LY294002 (Ly) was injected (40 mg/kg BW, i.p.) 20 min prior to insulin injection (Ins, 1 U/kg BW) via the inferior vena cava. Myocardial mitochondria were isolated and mitochondrial proteins were used for immunoblotting. *In vivo* inhibition by LY294002 suppressed phosphorylation of Akt (upper panel). LY294002 did not inhibit the Akt translocation to mitochondria (lower panel). **B:** Mitochondria *in vitro* import assay. Myocardial mitochondria were isolated from overnight-fasted mice without insulin stimulation. To collect activated Akt, mitochondria-free cytosolic protein fractions were isolated from insulin-stimulated myocardium. The activated Akt was incubated with mitochondria for 2 to 60 min and the reaction mixtures were incubated with Proteinase K to remove extra-mitochondrial proteins and resolved with SDS-PAGE for immunoblotting. The imported p-Akt was detected after 2 min of incubation. OPA1 and MnSOD respectively served as markers for intermembrane space (IMS) and matrix. TOM 20 is exposed on the outer membrane and was digested by proteinase K as anticipated, thus ruling out contamination from the cytosolic fraction. Insulin-stimulated cytosolic protein preparations were used as a control (C), indicating the absence of mitochondrial protein contamination in the cytosolic protein preparations. Whole cell lysate (WCL) from insulin stimulated myocardium was used as a positive control. Electrochemical-gradient ($\Delta\Psi$) dissipated mitochondria, by adding AVO (AV), contained less imported p-Akt. The bar graph represents the effect of AVO, summarized from three independent experiments, on *in vitro* p-Akt import. **C:** Time-course of p-Akt translocation to outer membrane, intermembrane space, and inner membrane of mitochondria. Mitochondrial subfractionation was performed as described in the Materials and methods section. p-Akt was present in the outer membrane (OM) and IMS after 2 min of insulin stimulation. p-Akt was detected in the inner membrane (IM) after, at least, 5 min of insulin stimulation. Cytochrome c, AIF and AK2 were used as IMS marker, and OPA1 and ATP synthase subunit d as IM markers. **D:** Translocated p-Akt reached the mitochondrial matrix. Control

and insulin-stimulated mitochondria were washed and resuspended in a swelling buffer (10 mM KH_2PO_4 , pH 7.4) and sonicated. The resulting preparations were centrifuged and the supernatants (matrix) and the pellets (membrane fraction, M + IMS) collected. Equal amounts of proteins were analyzed with immunoblotting. AIF, porin, and MnSOD, respectively, were used as markers for IMS, membranes, and matrix.

Fig. 2.

Insulin stimulated differential Akt isoform translocation. A: Translocation of Akt isoforms in myocardial mitochondria. After overnight fasting, mice were injected with insulin or vehicles. Basal (-) and insulin-stimulated (+) mitochondria were isolated from myocardium and mitochondrial proteins extracted for western blots with specific Akt1 or Akt2 antibodies. B, C, D, and E: Insulin stimulated Akt isoform translocation to mitochondria in diabetic myocardium. The abundance of Akt isoforms in mitochondria from control (Con), streptozotocin-induced (STZ) and diet-induced (high fat/fructose, HFF) diabetic mice was analyzed with immunoblots. Representative blots were shown in B (Akt1) and C (Akt2). Densitometry analysis from 6 to 7 independent experiments is presented in the bar graph D (Akt1) and E (Akt2), as percentage of control basal.

Fig. 3.

Insulin induced accumulation of p-Akt1 in mitochondria. Cardiomyocytes were serum-deprived overnight and stimulated with insulin for 10 min. The cells were fixed with 4% formaldehyde for 30 min at room temperature. After washing, cardiomyocytes were treated with 0.05% saponin for 20 min and blocked with 10% normal sera for 30 min. The fixed cells were stained with anti-p-Akt1 antibodies (A and B) and Hsp90 antibodies (B), mitochondria were visualized with mitotracker when indicated and nucleus was counterstained with DAPI. The immunofluorescence images were acquired with Zeiss AxioPlan II fluorescence microscope.

Fig. 4.

Mitochondrial Akt isoforms in basal and insulin-stimulated myocardium of wildtype (WT), Akt1^{-/-} (A1KO) and Akt2^{-/-} (A2KO) mice. Whole cell lysate (WCL) was used as a control for immunoblotting. Insulin or vehicles were injected when indicated as described in the Materials and methods section. A: The effects of insulin stimulation on mitochondrial Akt1 translocation. Akt1 was absent in A1KO mice. In the WT and A2KO, insulin stimulation increased the abundance of Akt1 in mitochondria. B: The effects of insulin stimulation on mitochondrial Akt2 translocation. Akt2 was absent in A2KO WCL and mitochondria. There was no Akt2 translocation to mitochondria in the WT mice. Significant compensatory increase of Akt2 protein was detected in the insulin-stimulated A1KO mitochondria. (C) Porin-normalized OXPHO Complex V activity in WT, A1KO and A2KO mitochondria. Basal Complex V activity was significantly lower in Akt1^{-/-} mitochondria, but basal Complex V activity was not attenuated in Akt2^{-/-} mitochondria. Insulin enhanced Complex V activity in all three groups (*p < 0.05 vs. basal; #p < 0.05 vs. WT basal; NS, not significant vs. WT basal). (D) Respiration P:O ratio of the mitochondria from WT, A1KO and A2KO myocardium. *p < 0.05 vs. WT.

Fig. 5.

Expression of a mitochondria-targeting, constitutively active Akt1 enhanced Complex V activity in cardiac muscle. Ad-mito-Akt and Ad-GFP were transduced to the myocardium of normal and diabetic rats as described in the Materials and methods section. Diabetes was induced by streptozotocin (STZ). **A:** Schematic illustration of Ad-mito-Akt construction. Mitochondrial targeting was achieved by attaching a mitochondrial targeting sequence of cytochrome *c* oxidase subunit 8A, whereas changing threonine 308 and serine 473 to aspartic acid (T308D and S473D) rendered Akt constitutively active. **B:** Myocardial cryosection showed diffuse overexpression of transgene (GFP) in the cardiac muscle cells transduced with Ad-GFP. **C:** Increased expression of Akt in the myocardium injected with Ad-mito-Akt. Mitochondria were isolated and proteins extracted for western blots. Porin served as loading controls. **D:** OXPHO Complex V subunit abundance was not altered in the myocardium transduced with Ad-mito-Akt. Complex V subunits were isolated with Complex V Immunocapture Kit and resolved with SDS-PAGE. The abundance of subunits from multiple samples was summarized in the bar graph. **E:** Transduction of Ad-mito-Akt increased mitochondrial OXPHO Complex V activity. 3 days after transduction with Ad-mito-Akt (mAkt) and Ad-GFP (GFP), myocardium was harvested and mitochondria isolated for Complex V activity analysis. **p* < 0.05 vs. control with Ad-GFP; #*p* < 0.005 vs. STZ-DM with Ad-GFP. **F:** ATP content. Cardiomyocytes were transduced with control vectors, Ad-mito-Akt (mAkt), or Ad-mito-dnAkt (mdnAkt) and the cells were analyzed for ATP content. **p* < 0.001, #*p* < 0.01 vs. control. **G:** Ad-mito-Akt increased phosphocreatine levels in cardiac muscle cells. Phosphocreatine (p-Cr) was analyzed with mass spectrometry as described in the Materials and methods section, and the ratios of p-Cr/ATP were determined in each sample. The relative content of p-Cr (*m/z*: 172) between different samples were normalized with UMP (*m/z*: 332) in the positive mode. **p* < 0.002 vs. Ad-GFP. **H:** Mitochondrial matrix calcium level. Cardiomyocytes were transduced with control vectors,

Ad-mito-Akt (mAkt), or Ad-mito-dnAkt (mdnAkt) and the cells were analyzed with rhod-2 AM. * $p < 0.01$ vs. control.