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Novel remodeling of the mouse heart mitochondrial proteome in response to acute insulin stimulation

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Abstract

Mitochondrial dysfunction contributes to the pathophysiology of diabetic cardiomyopathy. The aim of this study was to investigate the acute changes in the mitochondrial proteome in response to insulin stimulation. Cardiac mitochondria from C57BL/6 mice after insulin stimulation were analyzed using two-dimensional fluorescence difference gel electrophoresis. MALDI-TOF MS/MS was utilized to identify differences. Two enzymes involved in metabolism and four structural proteins were identified. Succinyl-CoA ligase [ADP forming] subunit beta was identified as one of the differentially regulated proteins. Upon insulin stimulation, a relatively more acidic isoform of this protein was increased by 53% and its functional activity was decreased by ∼32%. This proteomic remodeling in response to insulin stimulation may play an important role in the normal and diabetic heart.

1. Introduction

Cardiovascular disease is a major cause of increased morbidity and mortality in diabetics [1]. A variety of stimuli over time in diabetic myocardium lead to ventricular dysfunction and development of diabetic cardiomyopathy (DCM). A sundry of mechanisms are thought to contribute to DCM and involve altered energy metabolism in response to insulin, increased levels of oxidative stress, decreased autophagy, dysregulated Ca^{2+} handling,

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activation of inflammatory and apoptotic pathways and many others. Mitochondria play a role in many of these processes and have been implicated as a key player in the development of heart disease in diabetics [2-6].

Under normal physiological conditions, the heart's high ATP demands are met primarily through the oxidation of fatty acids by mitochondria [7]. However, glucose metabolism becomes relatively more important during such insults as ischemia, pressure overload and changes in substrate availability [7, 8]. Importantly, it is the response to insulin by the myocardium during these normal and pathological states that is thought to be central to the heart's metabolic flexibility and dynamic functional capacity [8, 9]. Additionally, mitochondrial proteins are involved in regulating these key biological processes and have been shown to demonstrate tissue-specific post-translational modifications [10]. However, the role of acute insulin on these differences remains to be established.

In this study, we investigated alterations in the mitochondrial proteome of the heart in the setting of acute insulin stimulation. Using inbred mice and controlling for nutritional status and diurnal variation, we analyzed the proteome of highly purified heart mitochondria of insulin stimulated animals in comparison to controls using two-dimensional fluorescence difference gel electrophoresis with subsequent identification of differentially regulated proteins using tandem mass spectrometry. We identified six proteins with a high level of confidence. ATP-specific succinyl-CoA synthetase subunit beta (A-SCS), one of the identified proteins and not previously known to be regulated by insulin, was shown to have diminished activity following insulin stimulation. Our studies demonstrate that acute insulin stimulation leads to mitochondrial protein remodeling that can contribute to changes in cardiac metabolism.

2. Materials and Methods

2.1. Materials

Human Insulin R (rDNA origin) was manufactured by Eli Lilly and Company (Indianapolis, IN). Mitochondria Isolation kit was purchased from Milteny Biotec (Auburn, CA). Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad (Hercules, CA). Anti-phospho Akt (Ser473) (D9E) antibody was purchased from Cell SignalingTechnology (Danvers, MA). Anti-SOD2 antibody (9E2BD2) was purchased from Abcam (Cambridge, MA). CyDye fluorescent dyes, pH 3-7 NL and pH 3-11 NL Immoboline DryStrips gels were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Precast gradient SDS-PAGE gels were purchased from JULE, Inc. (Milford, CT). α-cyano-4-hydroxycinnamic acid was purchased from ProteoChem (Loves Park, IL). All other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Nunc brand, clear, flat bottom, 96-well microplates were purchased from Thermo Fisher Scientific (Waltham, MA). ZipTips$_{C18}$ were purchased from EMD Millipore (Billerica, MA).

2.2. Animals

All studies were approved by the Institutional Animal Care and Use Committee at The University of California at Irvine and comply with the National Institutes of Health guidelines. All C57BL/6 mice used were male, 6-8 weeks of age at the time of experiments,

and purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Mice were fasted overnight for 16-18 hours overnight prior to experiments and were euthanized by cervical dislocation. For studies involving acute insulin stimulation, mice were injected intraperitoneally with either insulin (1U/Kg body weight) or phosphate-buffered saline (PBS)[11]. To assess the effects of a carbohydrate load, mice were orally gavaged with either glucose (2g/kg body weight) or water[12].

2.3. Measurement of Glucose Levels

Blood was collected by tail vein sampling and blood glucose levels were measured using an ACCU-CHEK Compact Plus (Roche Diagnostics, Indianapolis, IN) automatic glucometer.

2.4. Mitochondrial Isolation

Harvested hearts were immediately rinsed in cold PBS to remove excess blood and then diced in mannitol/sucrose buffer (MSB). MSB is comprised of 225mM mannitol, 75mM sucrose, 5mM HEPES, 0.5% BSA, fraction V, 1× SigmaFAST protease inhibitor cocktail (S8830), 20mM NaF, and 2mM Na3VO4. Diced tissue was then transferred to an ice-cold Dounce tissue grinder tube containing MSB supplemented with 1mM ATP and 200U/mL collagenase, type I. A polytetrafluoroethylene (PTFE) coated pestle attached to a controlled stirrer set to 500rpm was used to dounce homogenize the tissue five times. Douncing was performed with the grinder tube in an ice-water bath. The sample was then centrifuged at 1000g for 10 minutes at 4°C to pellet intact cells and nuclei. For proteomic analysis, the supernatant was then collected and MACS Technology was employed for mitochondria isolation as per the manufacturer's protocol (Miltenyi Biotec) [13]. For determining succinyl-CoA activity, the supernatant was collected and mitochondria were pelleted at 10,000g for 10 minutes at 4°C and then washed twice in MSB.

2.5. Western Blot

Briefly, 50ug (isolated mitochondria using MACS Technology) of protein as per a Bradford assay (Bio-Rad) was separated on a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene diflouride membrane, and incubated in blocking solution (5% BSA in 20 mM Tris–HCl [pH7.6], 137 mM NaCl, and 0.1% Tween 20(TBS-T)) for 1 hour at room temperature and then incubated with primary antibody overnight at 4°C [14, 15]. Membranes were then washed three times with TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 90 minutes. Membranes were washed another three times and signals were detected using an Immun-Star WesternC Chemiluminescence Kit (Bio-Rad) and digitally captured (VersaDoc Imaging System, Bio-Rad).

2.6. Sample Preparation for 2-D DIGE

Mitochondrial pellets were re-suspended in 25μl of DIGE sample labeling buffer (7M urea, 2M thiourea, 30 mM Tris base, 4% CHAPS, pH 9) and incubated at room temperature for 10 min prior to protein quantification by the Bradford assay (Bio-Rad). Mitochondrial extracts were minimally labeled with N-hydroxy-succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3 and Cy5 according to the manufacturer's instructions. Briefly, 400pmoles of these

dyes were used per 50ug of protein with the concentration of the protein lysate at 5mg/ml. Each sample group (PBS,insulin treated or glucose fed) was comprised on an equal amount of protein from each individual of the group. Samples were labeled for 30 minutes in the dark on ice and the labeling reaction was subsequently quenched with the addition of 1ul of 10mM L-lysine on ice for 10 minutes [16]. The labeling strategy was as follows: control and treated samples were labeled with Cy3 and Cy5 and for normalization the same amount of protein from a pool of all samples was labeled with Cy2. Differentially labeled samples were mixed prior to rehydration loading.

2.7. IPG-strip Rehydration, IEF and 2-D Gel Electrophoresis

Mixed labeled samples were made up to 425 μl with DeStreak-rehydration solution (GE-Healthcare), including 0.5% IPG buffer 3–11 NL and then transferred to a rehydration tray and an Immobiline Dry Strip pH 3-11NL was placed gel side down into the channel. The strip was then overlayed with mineral oil and allowed to rehydrate for 20 hours at room temperature in the dark. The first dimension was run an Ettan IPGphor3 (GE Healthcare) and started at 100V and continued until the current was less than 5uA per strip (approximately 12hrs) to desalt the sample and to ensure reaching the highest voltage. Isoelectric focusing was continued as follows: 1 hour at 500V, a gradient voltage for 1 hour to 1000V, a gradient voltage for 3 hours to 8000V, 3 hours at 8000V, a gradient voltage for 3 hours to 10000 V and a final step at 10000V for 2 hours. Prior to the second dimension, the gel strips were equilibrated and disulfide bonds were reduced and alkylated in buffer (6 M urea, 2% SDS, 30% glycerol, and 50 mM Tris–HCl, pH 8.6) containing 1% (w/v) DTT for the reduction reaction and then 2.5% (w/v) iodoacetamide for the alkylation reactions for 15 minutes at room temperature. Strips were then rinsed with 2× electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 0.2% SDS) and sealed onto 4-15% SDS-PAGE gels (JULE, Inc) with 1% (w/v) agarose solution in 1× SDS-PAGE buffer containing a trace of bromophenol blue (0.002% w/v). Electrophoresis was performed in 1× SDS-PAGE buffer at 20°C with an Ettan Dalt-six system (GE Healthcare) applying 10mA/gel for one hour and then 40mA/gel until the blue front reached the bottom of the gel. Preparative gels were prepared as described above with the exception of DryStrip gels being rehydrated with 500 μg of unlabeled protein extracts and gels being stained with Coomassie Brilliant Blue G-250 overnight as per a previously described protocol [17]. Protein spot matching between analytical and preparative gels and their excision from the gel were performed manually.

2.8. 2-D DIGE Image Analysis

The Cy2, Cy3 and Cy5 labeled images were individually acquired on a Typhoon FLA 9500 (GE Healthcare) at the excitation/emission wavelengths of 473/530 nm, 532/570 nm, 635/665 nm, respectively. Two dimensional gels were analyzed using DeCyder 2D software (version 7.0, GE Healthcare). To optimize comparative gel analysis, spot detection was performed after background subtraction and normalization of a set of images from the same gel. Gels were analyzed in the DIA module with exclusion selection criteria on spot volume (<10000) and maximum slope (>1.0) then loaded into the BVA module for comparative analysis across multiple gels to identify differential spots between the control and insulin stimulated group (abundance ratios of ≥+1.5 or ≤-1.5 fold and p-value ≤ 0.05).

2.9. In-Gel Digestion and Protein Identification using MALDI TOF MS and MS/MS

In-gel digestion of spots with trypsin was as previously described [18]. Briefly, excised gel pieces were washed once in 25mM ammonium bicarbonate, followed by two washes in 50%ACN/25mM ammonium bicarbonate and finally dehydrated in 100% ACN and subsequently dried to completion under vacuum while centrifuged. Gel pieces were incubated with trypsin (Sigma) at a concentration of 12.5ng/ul. Peptides were recovered with 60% ACN/5% formic acid and resuspended in 0.1% formic acid. All samples were desalted using $ZipTips_{C18}$ as per the manufacturer's protocol and eluted directly in an α-cyano-4-hydroxycinnamic acid saturated solution (60% ACN/5% formic acid) onto a MALDI target plate with the dried droplet method. Spectra were acquired on an AB SCIEX TOF/TOF 5800 system with spectra from MALDI-TOF acquired in positive ion reflector mode and MS/MS spectra acquired in 1-kV positive mode. A mass accuracy tolerance of 30 ppm for precursors and 0.3 Da for fragments were permitted for tryptic mass searches of *Mus musculus* proteins in the UniProt database using the Paragon algorithm (ProteinPilot version 4.0; ABSCIEX). Positive protein identifications were made if more than two peptides with 95% confidence, as per the Paragon algorithm scoring system, mapped to the same protein.

2.10. Succinyl-CoA Ligase Activity Assay

The *in vitro* assay for ADP-forming succinyl-CoA ligase activity was conducted based on a protocol previously described [19]. To ensure an adequate amount of protein for testing, mitochondrial protein fractions from four mice were pooled together for each experimental group. The assay was performed at 37°C in a volume of 200uL in a microplate. Mitochondrial extracts were resuspended in 0.4% (w/v) CHAPS in 20mM potassium phosphate buffer (pH 7.2) and the protein amount was quantified by the Bradford assay. The protein level was then adjusted to the indicated concentrations in the same buffer. An assay mixture of 50mM potassium phosphate buffer, 10mM MgCl2, 1mM succinyl-CoA, and 2mM ADP was then prepared. We found that all lots of succinyl-CoA contained reducing agents capable of reacting with DTNB. As such, DTNB at a final concentration of 0.2mM in 50mM potassium phosphate buffer solution (pH 7.2) was added to the assay mixture and incubated at 37°C for three minutes in a microplate reader to allow for the near completion of this non-enzymatic reaction prior to the addition of the mitochondrial extract. The desired amount of protein was then added to the microplate and the formation of thionitrobenzoate (TNB) was followed at 412nm on a BioTek Synergy HT multimode microplate reader (Winooski, VT). Rates were corrected by subtracting the rate when ADP was deleted. Controls demonstrated that the activity was dependent on $MgCl_2$ and ADP.

3. Results

3.1 Time Course of Acute Insulin Stimulation on the Heart

To maximize the sensitivity of the downstream proteomic analysis, a time course of the effects of insulin stimulation on the heart was performed. Previous work has demonstrated the translocation of Akt in its active, phosphorylated state to the mitochondria upon stimulation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway [20]. As such, we used Western blot analysis of p-AKT in heart mitochondrial fractions subsequent to insulin

stimulation as an indicator of the effectiveness of the stimulation (Figure 1). Control mice were injected with PBS and the time points of 10, 20, and 30 minutes post insulin injection were used for the time course. The sample at each time point is from an equal pool of protein comprised of four individual mice. As a loading control, p-AKT signal intensities were normalized to that of a mitochondrial matrix protein (Mn-SOD2). A maximal increase in p-Akt levels was detected 10 minutes after injection with insulin. This level was 4.1 fold greater than the basal level. Given the rapid and robust accumulation of p-Akt in the mitochondrial fraction at 10 minutes post injection and subsequent decline, we decided to analyze the acute effects of insulin on the heart mitochondrial proteome at this time point.

3.2 2D Electrophoresis-Heart Mitochondrial Proteomics of Acute Insulin Stimulation

We performed a comparative analysis of heart mitochondrial protein profiles of control and insulin stimulated samples using 2D-DIGE (Figure 2). Each gel contained a Cy3 and Cy5 labeled extract from a pooled sample of four individuals of the same experimental group that was co-resolved with a Cy2 labeled internal standard consisting of a pooled sample comprised of all individuals. This enabled every protein spot to be measured relative to the cognate signal within a gel, as well as, allowed for normalization to the control pool and comparison of these ratios between biological replicates across four 2-D DIGE gels. As per DeCyder software, approximately 2000 protein spots were detected on each gel. A comparison of heart mitochondrial proteins from insulin stimulated mice (n=8) with those from controls (n=8) revealed differential expression of 9 protein spots between the two groups. This was based on a statistical cut off point of $p \leq 0.05$ and an intensity fold change ≤ -1.5 or $\geq +1.5$. All 9 protein spots identified were up-regulated in the insulin group in comparison to the control. These spots, along with others, were subsequently excised from a Coomassie Brilliant Blue G-250 stained preparative gel and identified through the use of tandem mass spectrometry and database interrogation. 3 of the 9 proteins were identified in multiple spots in the same molecular weight range and thus likely represent post-translational modifications or unique isoforms. As a result of this, 6 proteins were identified as differentially regulated (Table 1). Surprisingly, only two of these proteins (ATP synthase subunit beta and ATP-specific succinyl-CoA synthetase subunit beta) localized to the mitochondria. Importantly, these results suggest that the TCA cycle and the electron transport chain in the heart may be tightly regulated by insulin.

3.3 ATP-specific Succinyl-CoA Synthetase Activity

As previous work has shown that insulin stimulation increases ATP synthase (Complex V) activity, we chose to validate the findings of the 2D-DIGE and to better understand the functional implications by performing a functional assay of ATP-specific succinyl-CoA synthetase activity[19]. We measured activity in the direction of succinyl-CoA to succinate with an assay based on a variation of Lambeth's protocol that accommodated for the variable amount of reducing agents found in different batches of succinyl-CoA. As this assay requires a relatively large amount of protein extract, the mitochondrial protein extract from four hearts was pooled for each group for each assay. Representative results from one of three trials are shown in Figure 3 and activity levels are expressed as a change in OD/μg of protein extract after correction for ADP independent activity. We observed an approximately 32% decrease (mean relative activity ± SD; 0.681 ± 0.062) in activity in

ATP-specific succinyl-CoA activity in heart mitochondria following stimulation with insulin in comparison to controls. To our knowledge, this is the first time this assay has been applied to murine samples and demonstrates a functional consequence on a TCA cycle enzyme in the heart upon insulin stimulation.

4. Discussion

The action of insulin on the heart significantly impacts its substrate utilization. Under normal physiological conditions, ∼90% of its energy requirements to supply the body with oxygenated blood are met through the oxidation of fatty acids. However, after feeding, when insulin levels rise, glucose is more readily transported into the myocardium and becomes the primary energy source. This transition is relatively swift and a multitude of regulated mechanisms contribute to the heart's flexible substrate usage. Ion fluxes, metabolite levels, end-product inhibition and post-translational modifications are all mechanisms by which this process is coordinated.

In this study, we used a 2-D electrophoresis approach to investigate the acute proteomic changes in heart mitochondria in response to insulin. A strength of this approach is the ability to identify relative changes in post-translational modifications. As such, we were able to identify post-translational changes in two mitochondrial proteins, ATP synthase beta and A-SCS. Additionally, we found a decrease in the activity level of A-SCS upon exposure to insulin. This is consistent with previous results that have demonstrated an increase of metabolites upstream of A-SCS in the TCA cycle in the setting of insulin receptor saturation and this in turn slows down the oxidation of fatty acids [7, 21]. Such a transition in substrate utilization reduces the generation of reactive oxygen species (ROS) and increases cardiac efficiency in terms of ATP produced/O2 consumption. This ability of the heart to preferentially oxidize glucose in the fed state subsequently reduces the potentially damaging effects of excess substrate. The carbon chains of isoleucine, methionine, threonine, and valine (gluconeogenic amino acids) enter the TCA cycle in the form of succinyl-CoA. Our system demonstrates that in the setting of insulin signaling, which is a surrogate for abundant blood glucose levels, the heart is able to devote less energy to the process of gluconeogenesis and further increase its metabolic efficiency. Additionally, recent work has demonstrated lysine succinylation of key enzymes of the TCA cycle and fatty oxidation [22, 23]. However, to date no lysine succinyltransferase has been identified and some have postulated that this is a non-enzymatic process. It is quite possible that the relative increase of succinyl-CoA in the insulin-stimulated state provides for an increase in the succinyl donor and hence regulatory post-translational modifications of key enzymes involved in metabolism.

A caveat of these findings is the utilization of a supra-physiological insulin stimulus that is commonly used in the field to study *in vivo* insulin signaling[24-26]. Such dosing is typically used to elicit a rapid, maximal and relatively sustained response to increase the sensitivity of downstream analyses. In the interest of studying heart mitochondrial protein changes under more physiological conditions, we performed similar proteomic analyses as described above after oral glucose feeding (2g/Kg body weight). In these experiments, designed to investigate glucose induced insulin secretion and subsequent proteomic changes,

we detected the differential regulation of only a single mitochondrial protein species (acyl carrier protein, mitochondrial was downregulated by 62%; Supplemental Figures 1-3, Supplemental Table 1). Such a result is not entirely surprising given that previous work has highlighted these dependencies given tissue specific and time course differences between and within tissues for such stimuli[27]. This interesting difference between direct insulin stimulation and carbohydrate loading indeed warrants consideration when researchers are planning their investigations.

Our work also demonstrated post-translational modifications of cytoskeleton proteins within our mitochondrial fractions. This is in the context of using a mitochondrial enrichment protocol that has previously been shown to significantly decrease the abundance of peroxisomal, microsomal, endoplasmic reticulum protein in comparison to more traditional approaches[28]. While the inclusion of these proteins is likely an artifact of cardiac mitochondrial isolation, it is possible that this may have some functional consequences as the hydraulic work of the heart has been shown to increase upon insulin stimulation[29]. The mechanism for this may in part be dependent upon the differential phosphorylation of these proteins. Interestingly, cardiomyopathies have previously been found in murine models in which characterized phosphorylation sites of tropomyosin alpha-1 chain and myosin regulatory light chain 2 have been mutated [30, 31]. It is quite possible that in states of insulin resistance the phosphorylation status at these sites is not properly regulated and contributes to cardiac dysfunction.

In conclusion, we have shown that acute insulin stimulation can rapidly induce cardiac mitochondrial protein remodeling that contributes to its highly regulated process of substrate utilization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Acute insulin stimulation caused remodeling of the mouse cardiac proteome
- Changes were found in metabolic and structural proteins
- Insulin stimulation decreased succinyl-CoA synthetase activity by 32%

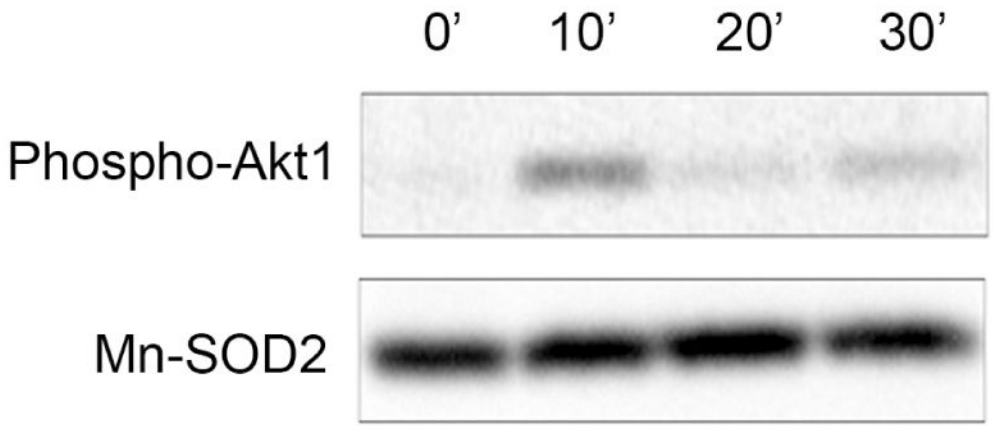

Figure 1.

Time course of phospho-Akt1 translocation to mitochondria upon in vivo insulin stimulation in the heart. Representative Western blots depicting phospho-Akt1 and Mn-SOD2 protein abundance in isolated heart mitochondria. Each time point is of a pool of mitochondria from 4 animals. Immunoblot with anti-Mn-SOD2 served as a loading control.

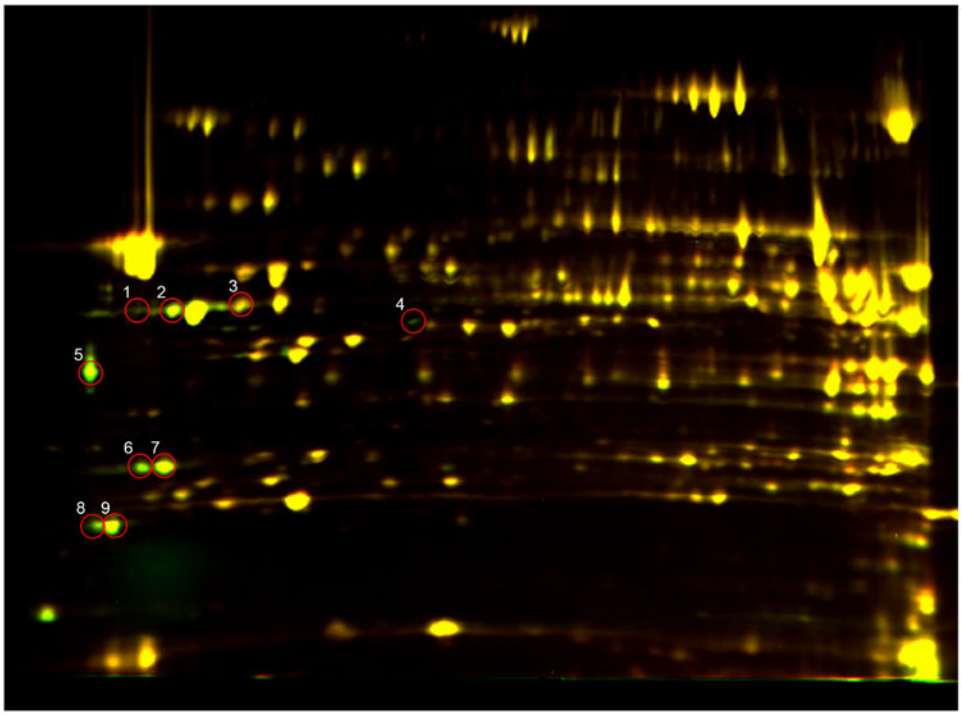

Figure 2.

Two-dimensional differential electrophoresis of insulin stimulated cardiac mitochondria. Overlay of insulin stimulated vs. control. 1) actin, alpha cardiac muscle 1/ATP synthase subunit β, mitochondrial; 2) actin, alpha cardiac muscle 1; 3) succinyl-CoA ligase [ADP-forming] subunit β; 4) actin, alpha cardiac muscle 1; 5) tropomyosin α-1 chain; 6) myosin light chain 3; 7) myosin light chain 3; 8) myosin regulatory light chain 2, ventricular cardiac muscle isoform; 9) myosin regulatory light chain 2, ventricular cardiac muscle isoform. Additional information on spot identities and differential expression is in Table 1.

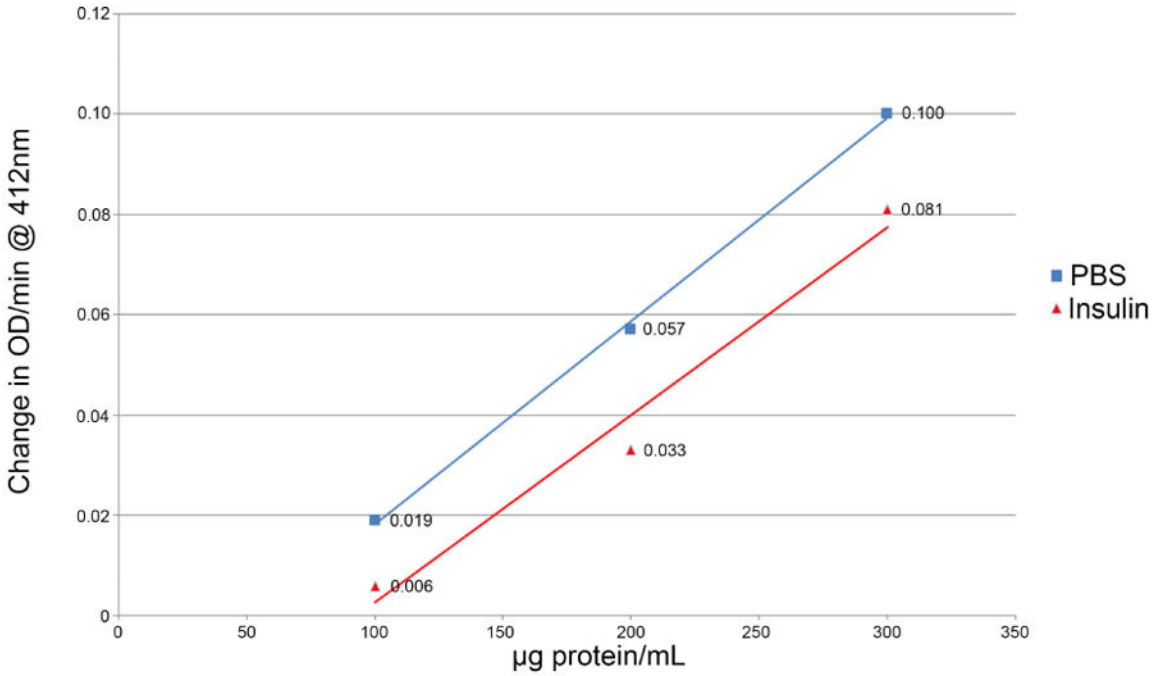

Figure 3.

Acute insulin stimulation decreases the activity of ATP-specific succinyl-CoA synthetase in heart mitochondria. Shown are representative rates of conversion of succinyl-CoA to CoASH in mitochondrial pools from 4 animals as monitored by the reduction of DTNB. Observed rates were corrected for the nonenzymatic hydrolysis of succinyl-CoA and done in parallel with controls that demonstrated activity to be dependent on MgCl2 and ADP.

Table 1

2D-DIGE Analysis. List of proteins identified in spots when comparing heart mitochondrial samples from control and insulin stimulated C57BL/6 mice. All variant protein spots had a fold cutoff change of ≤-1.5 or ≥ 1.5 and a *p*- value <0.05 in both replicates. Mean fold change is for insulin treated mice in comparison to control mice (PBS treated). The spot numbers in the table refer to the spot numbering in Figure 2.

Spot #	Protein Name	UniProt ID	Mean Fold Change	*p* -value
1	actin, alpha cardiac muscle 1; ATP synthase subunit β, mitochondrial	ACTC_MOUSE; ATPB_MOUSE	+1.62	0.071
2	actin, alpha cardiac muscle 1	ACTC_MOUSE	+1.83	0.03
3	succinyl-CoA ligase [ADP-forming] subunit β	SUCB1_MOUSE	+1.53	0.0029
4	actin, alpha cardiac muscle 1	ACTC_MOUSE	+2.20	0.0072
5	tropomyosin α-1 chain	TPM1_MOUSE	+1.95	0.034
6	myosin light chain 3	MYL3_MOUSE	+1.84	0.002
7	myosin light chain 3	MYL3_MOUSE	+1.85	0.0061
8	myosin regulatory light chain 2, ventricular cardiac muscle isoform	MLRV_MOUSE	+1.68	0.024
9	myosin regulatory light chain 2, ventricular cardiac muscle isoform	MLRV_MOUSE	+1.83	0.0054