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Proteomic Analyses of Transgenic LQT1 and LQT2 Rabbit Hearts Elucidate an Increase in Expression and Activity of Energy Producing Enzymes

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Abstract

Various biochemical and genomic mechanisms are considered to be hallmark of metabolic remodeling in the stressed heart, including in the hypertrophied and failing heart. In this study, we used quantitative proteomic 2-D Fluorescence Difference In-Gel Electrophoresis (2-D DIGE) in conjunction with mass spectrometry to demonstrate differential protein expression in the hearts of transgenic rabbit models of Long QT Syndrome 1 (LQT1) and Long QT Syndrome 2 (LQT2) as compared to littermate controls (LMC). The results of our proteomic analysis revealed upregulation of key metabolic enzymes involved in all pathways associated with ATP generation, including creatine kinase in both LQT1 and LQT2 rabbit hearts. Additionally, the expression of lamin-A protein was increased in both LQT1 and LQT2 rabbit hearts as was the expression of mitochondrial aldehyde dehydrogenase and desmoplakin in LQT1 and LQT 2 rabbit hearts, respectively. Results of the proteomic analysis also demonstrated down regulation in the expression of protein disulfide-isomerase A3 precursor and dynamin-like 120 kDa protein (mitochondrial) in LQT1, and of alpha-actinin 2 in LQT2 rabbit hearts. Up regulation of the expression of the enzymes associated with ATP generation was substantiated by the results of selective enzyme assays in LQT1 and LQT2 hearts, as compared to LMC, which revealed increases in the activities of glycogen phosphorylase (+50%, +65%, respectively), lactate dehydrogenase (+25%, +25%), pyruvate dehydrogenase (+31%, +22%), and succinate dehydrogenase (+32%, +60%). The activity of cytochrome c-oxidase, a marker for the mitochondrial function was also found to be significantly elevated (+80%) in LQT1 rabbit hearts as compared with LMC. Western blot analysis in LQT1 and LQT2 hearts compared to LMC revealed an increase in the expression of very-long chain-specific acyl-CoA dehydrogenase

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(+35%, +33%), a rate-limiting enzymes in β -oxidation of fatty acids. Collectively, our results demonstrate similar increases in the expression and activities of key ATP-generating enzymes in LQT1 and LQT2 rabbit hearts, suggesting an increased demand, and in turn, increased energy supply across the entire metabolic pathway by virtue of the upregulation of enzymes involved in energy generation.

Keywords

2-D DIGE; proteomics; mass spectrometry; Long QT syndrome; metabolic remodeling; energetics

1. Introduction

Long QT syndrome (LQTS) is an autosomal dominant disease characterized by prolongation of the QT interval in the surface ECG and spontaneous polymorphic ventricular tachycardia (VT) [1-4]. A prolonged QT interval increases the likelihood of ventricular arrhythmia and sudden cardiac death [5]. At least 12 different forms of LQTS and the mutations in genes associated with these syndromes have been identified [6]. LQT1 and LQT2 are the most common type, representing 30-35% of all cases. LQT1 is caused by mutations in the gene *KCNQ1* which encodes the voltage-gated potassium channel KvLQT1; LQT2 involves mutations of the *human ether-a-go-go related gene* (HERG or *KCNH2*). In both LQT1 and LQT2, a dominant negative mechanism has been suggested for many of these mutations [7-10].

The regulation of gene expression, transcription, mRNA translation, protein expression, protein processing, subunit assembly, membrane transport, protein assembly, and post-translational regulation also has the capacity to mediate the remodeling of ion-channel expression and function [11]. Numerous cardiac abnormalities, including LQTS, result in arrhythmogenic remodeling, which involves alterations in ion channel and transporter expression, regulation, and is associated with a wide array of important protein partners [12].

Recently, cardiovascular proteomics has proved to be a potential tool in elucidating the many proteins and associated pathways that contribute to hypertrophic processes and various types of cardiac dysregulation [13, 14]. Proteomic studies have also been used to characterize global changes in cardiac protein expression in response to ischemia/reperfusion injury [15] in rabbit hearts, as well as in rat hearts [16], myocardial infarction [17, 18], pacing-induced heart failure in the dog [19-21] and in rabbits [22], and bovine dilated cardiomyopathy [23]. However, little is known about global differential protein expression in LQTS hearts.

Two-dimensional gel electrophoresis (2-DE) is a proteomic tool powerful in elucidating differential protein expression and post-translational modifications. 2-D DIGE is an improvement of 2-DE technology that improves gel reproducibility, minimizes alignment issues, and allows better differential protein expression analysis among samples. In 2-D DIGE, samples are labeled prior to electrophoresis with resolvable fluorescent dyes: Cy2, Cy3, and Cy5. Samples are then mixed prior to electrophoresis and resolved on the same gel

[24]. Several previous studies have employed 2-D DIGE to identify disease markers [25] specific to esophageal cancer [26] and, Alzheimer's disease [27]; to study the molecular responses induced by the activation or inhibition of signaling pathways [28]; to characterize proteins differentially expressed in response to growth factor [29]; and more importantly, in the area of cardiovascular proteomics, to elucidate differential protein expression in various cardiac diseases in human as well as animal models [16, 18, 20, 22, 30-33].

We have recently created two transgenic rabbit models for LQT1 and LQT2 by over-expressing in the heart the human loss-of-function pore mutants of KvLQT1 (KvLQT1 Y315S) or HERG (HERG G628S), respectively [4, 34], mimicking the human phenotypes of QT prolongation: prolonged action potential duration (APD) and increased calcium load/prolonged Ca^{2+} transient. We hypothesized that these phenotypes might play an important role in the global differential protein expression in these transgenic rabbit hearts. To address this, we have undertaken a proteomic 2-D DIGE approach in conjunction with mass spectrometry. Herein, we report the differential upregulation of the expression as well as activity of the metabolic enzymes in LQT1 and LQT2 rabbit hearts, which might suggest that metabolic remodeling compensates for the prolongation of the APD and increased calcium load/prolonged Ca^{2+} transient.

2. Materials and methods

2.1. Animal model

All animal experiments were performed in accordance with the local guidelines of the institutions and only after approval by the Institutional Animal Care and Use Committee (IACUC), also in accordance with the Institute for Laboratory Animal Research (ILAR) Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication #85-23; Revised 1996). The proteomic studies were performed in adult male and age-matched New Zealand white rabbits from, LQT1 (n=3) or LQT2 (n=3) lines and LMC (n=3) were used as controls.

2.2. Reagents

The following antibodies were used: anti-very long chain specific-acyl-CoA dehydrogenase (VLCAD) monoclonal antibody and anti mitochondrial uncoupling protein 2 (UCP2) monoclonal antibody were from Abcam; anti-pyruvate kinase goat polyclonal antibody was from Novus Biologicals; and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Sigma. The HRP-conjugated secondary antibodies for immunoblotting experiments were from Zymed Laboratories.

2.3. Sample preparation

Heart tissue samples (i.e., 250 mg of each sample from left ventricle-free wall) from transgenic rabbits were homogenized in homogenization buffer (7 M urea, 2M thiourea, 4% CHAPS, 1% Triton X-100, 10 mM Tris pH 8.5) containing 1 mM PMSF, protease inhibitors, and phosphates inhibitors. The homogenates were incubated for 1 h at 4° C by end-over-end mixing to ensure the efficient solubilization of the membrane proteins. The homogenates were centrifuged at $10,000 \times g$ for 15 min, and the supernatants representing

the total protein sample were taken for further analysis. Prior to labeling, the samples were cleaned using 2-D Clean-Up Kit (GE Healthcare). The protein concentration was determined using the 2-D Quant Kit as described by the manufacturer (GE Healthcare). Protein samples were dissolved in homogenization buffer to give stock solutions with a final concentration of 2-4 mg/ml. Initially, we attempted to dissolve the protein samples to a final concentration of 5-10 mg/ml using a 2-D Clean-Up Kit (GE Healthcare) in the sample buffer. However we found that the sample did not dissolve completely at this high concentration. Hence, the volume of the buffer was increased in order to dissolve the samples completely. 250 mg of tissue from each sample was taken for the preparation of total tissue extract for 2-D DIGE analysis, cytosolic preparation and mitochondrial preparation, respectively.

2.4 Labeling of samples

Protein samples were adjusted to pH 8.5 by addition of 50 mM NaOH. Samples were then labeled using Cy2, Cy3, and Cy5 dyes (CyDye DIGE Fluor minimal dyes; GE Healthcare). Cy dyes reconstituted in anhydrous dimethylformamide (DMF) were added to the protein samples in a ratio of 200 p mol CyDye per 50 µg protein. The samples from LMC were labeled with Cy3, and those from LQT1 or LQT2 rabbit hearts were labeled with Cy5. Standards were prepared by pooling equal amounts of protein from LMC and LQT1 or LQT2 and labeling with Cy2. Protein labeling was achieved by incubation at 4° C in the dark for 30 min. The reaction was quenched by further incubation with 10 mM lysine (1 µl per 200 p mole dye) followed by incubation at 4° C for 10 min in the dark.

2.5 Isoelectric Focusing (IEF)

Labeled proteins were first separated by IEF. Prior to subjecting the samples to IEF, labeled protein samples were mixed with an equal volume of 2 × sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 130 mM DTT, 2% v/v Pharmalytes 3-10). Samples were loaded onto IEF strips (Immobiline Dry Strips, pH 3-10 NL; GE Healthcare) by rehydration loading. Briefly, 150 µg protein sample per gel (50 µg from LMC plus 50 µg from LQT1 or LQT2 rabbit heart plus 50 µg of the internal pooled standard) were mixed with rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 1% Pharmalyte (pH 3-10), 2 mg/ml DTT] in final volume of 500 µl, and Immobiline IEF Dry Strips were rehydrated overnight, overlaid with mineral oil in an Immobiline Dry Strip reswelling tray (GE Healthcare). For each experiment, four Immobiline IEF Dry Strips were rehydrated with the protein samples. IEF strips were focused using 3-mm wide, water-soaked paper electrode pads on Ettan IPGphor 3 IEF system (GE Healthcare) for a total of 52,500 Vh at 20° C in four steps: 500 volts for 1 hr, linear ramping to 1000 V for 800 Vh, linear ramping to 10000 V for 16, 500 Vh and 10000 V for 34,700 Vh (setting a limit of 75 µA/strip). After IEF, the strips were equilibrated with 15-20 ml equilibration buffer A (6 M Urea, 75 mM Tris-HCl pH 6.8, 30% glycerol, 2% SDS and 10 mg/ml DTT) on a rocking table for 15 min, followed by 15 ml equilibration buffer B (6 M Urea, 75 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS, and 25 mg/ml iodoacetamide) for another 15 min.

2.6. SDS-PAGE

The strips were then inserted and run on 10% acrylamide isocratic Laemmli gels [35] that were subjected to electrophoresis in an Ettan DALTsix apparatus (GE Healthcare). Gels

were run at 1W per gel constant power at 20° C until the bromophenol blue dye front ran off the bottom of the gels.

2.7. Image acquisition

Gels were scanned between low-fluorescence glass plates using the Typhoon-9410 laser scanner (GE Healthcare). All gels were scanned at 100 µm resolution. Cy2 images were scanned using a 488-nm laser and a 520-nm BP40 emission filter. Cy3 images were scanned using a 532-nm laser and a 580-nm BP30 emission filter, and Cy5 images were scanned using 633-nm laser and a 670-nm BP30 emission filter. ImageQuant TL Version 7.0 software (GE Healthcare) was used to crop images to identical size by removing areas extraneous to protein spots prior to analysis. The gels were fixed in 50% methanol and 10% acetic acid and silver stained using Silver Stain Plus kit (Bio-Rad Laboratories).

2.8. DIGE Image analysis

DIGE images were analyzed using DeCyder 7.0 software (GE Healthcare). Spot detection was performed using batch processor function with an estimated number of spots set at 10,000. Four DIGE gels from each experiment were processed. DIGE images were further analyzed using the DeCyder BVA (biological variation analysis) module. Approximately 3,500 spots were detected on each gel. Protein spots exhibiting a statistical difference ($p < 0.05$) between LQT1 or LQT2 and LMC were picked for identification.

2.9. In-gel Protein Digestion and LC-MS/MS Analysis

Selected protein spots were excised from gels and digested in-gel with trypsin as described [36] with some modifications. Briefly, spots were destained using destaining reagents (Invitrogen), followed by washing twice with 50% acetonitrile in 25 mM ammonium bicarbonate (NH_4HCO_3). Protein spots were incubated with 200-500 µl of acetonitrile for 10 min at room temperature and spun down, and all the liquid was removed. The protein spots were next reduced with 10 mM DTT in 25 mM NH_4HCO_3 at 56°C for 30-45 min, alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 at room temperature for 1 h in the dark, and incubated with 500 µl of acetonitrile for 10 min to shrink spots. Protein spots were dried at room temperature and rehydrated with 30-50 µl of trypsin buffer (13 ng/µl trypsin in 25 mM NH_4HCO_3), incubated at 4°C for 2 hrs, and covered with an adequate volume of 25 mM NH_4HCO_3 . After overnight digestion at 37°C, the reaction was stopped by adding ~50 µl of acetonitrile. Peptides were extracted twice with solution containing 50% acetonitrile and 1% acetic acid, and the extracted digests were vacuum dried.

Tryptic peptides were fractionated on a reversed phase column and introduced directly onto a LTQ (“linear trap quadrupole”) mass spectrometer via electrospray ionization. Full mass spectrometry (MS) scans were followed by data-dependent acquisition of MS/MS spectra for the five most abundant ions. Peptide identifications were made via database matching with the program SEQUEST [37]. The search database contained sequences from the SwissProt database. A list of reversed sequences was created from these entries and appended to them for database searching so that false discovery rates could be estimated [37].

2.10. Preparation of Cytosol and Western Blot analysis

LV-free wall samples (250 mg each) were homogenized in homogenization buffer (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1 mM DTT, 10% glycerol, pH 7.5) containing protease inhibitors (Roche Diagnostics) using Dounce homogenizer. The homogenate was centrifuged at $15,000 \times g$ for 10 minutes at 4°C . The pellet was discarded and the supernatant was centrifuged at $100,000 \times g$ for 60 minutes at 4°C to obtain the cytosolic fraction. Western blot analysis was carried out as described previously [38]. Briefly, protein samples were heated in SDS sample buffer, and the proteins were resolved by electrophoresis on 10% SDS-PAGE gels. Proteins were transferred electrophoretically onto a nitrocellulose membrane and immunoblotted with specific antibodies (1-2 $\mu\text{g/ml}$) for 1 h. The blots were incubated with horseradish peroxidase-conjugated secondary antibody at 1:10,000 for 1 h. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL; Pierce). GAPDH was used as loading control. For quantification, Image J (NIH) software was used with the GAPDH signal utilized to correct for loading errors by calculating the ratio of the target protein intensity/GAPDH intensity.

2.11. Enzyme assays

The glycogen phosphorylase assay was based on the incorporation of [$\text{U-}^{14}\text{C}$] glucose 1-phosphate into glycogen as described previously [39]· [40]. Briefly, to assay for total glycogen phosphorylase activity, 30 μl of the diluted (diluted with 50 mM MES buffer, pH 6.1, containing 50 mM KF and 60 mM β -mercaptoethanol) total protein from the LV free wall was added to 60 μl of reaction mixture containing 200 mM KF, 100 mM glucose 1-phosphate- ^{14}C (0.0025 $\mu\text{Ci}/\mu\text{mole}$, Perkin Elmer), 1% glycogen (rabbit liver), and 3 mM 5'-AMP. The assay mixture was adjusted to pH 6.1[41]. The reaction mixture was incubated at 30°C for 20 min. A 75 μl aliquot was then removed and spotted on Whatman 31 ET filter paper. The filter paper was dried for about 30 s, washed twice in 66% ethanol for 20 min each, washed once with acetone for 2-3 min, dried at room temperature, and counted in a liquid scintillation system. The activity was expressed as n mol of glucose incorporated into glycogen per min per mg of protein. The lactate dehydrogenase activity was measured at 340 nm using a direct, plate-based, colorimetric reaction that converts lactate and NAD^{+} to pyruvate and NADH. The production of NADH was monitored by measuring the increase in absorbance of the reaction at 340 nm over 5 min according to the kit instructions (ID Labs Biotechnology, Inc.).

Pyruvate dehydrogenase (PDH) activity was determined by using the pyruvate dehydrogenase enzyme activity microplate assay kit (MitoSciences, Eugene, Oregon). Briefly, the PDH enzyme from the samples was immunoprecipitated within the wells of the microplate, and PDH activity was determined by following the reduction of NAD^{+} to NADH, coupled to the reduction of a reporter dye that yielded a yellow-colored product whose concentration was monitored by measuring the increase in absorbance at 450 nm.

Mitochondrial preparation from the heart tissue samples (250 mg) was performed using the mitochondrial isolation kit (Sigma, Saint Louis, Missouri). Succinate dehydrogenase activity in the mitochondrial preparations was assayed by employing $\text{K}_3\text{Fe}(\text{CN})_6$ as the artificial electron acceptor instead of FAD, and the rate of decrease in the concentration of K_3Fe

(CN)₆ was monitored at 420 nm at 30°C using an assay kit (Redoxica, Little Rock, Arkansas). Cytochrome c oxidase (complex IV) activity was determined by monitoring the decrease in absorbance at 550 nm upon oxidation of ferrocytochrome c to ferricytochrome c at room temperature using an assay kit (Sigma, Saint Louis, Missouri). The intactness and purity of the mitochondrial preparation was checked against the kit instructions. The mitochondrial preparation so obtained was more than 95% pure.

2.12. Experimental design and statistical methods

Three biological replicates were chosen for each phenotype, and within each biological replicate, four technical replicates were performed. Briefly, for each group/biological replicate, we ran gels in quadruplicate (i.e. four IPG strips and eventually four SDS-PAGE gels) at the same time. Each IPG strip and eventually a SDS-PAGE gel represented a technical replicate. The gel replicates were run in quadruplicate in order to create four data points for the purpose of statistical analysis. Statistical analysis was performed using the analysis of variance and student's t-test. Based on the quantitative analysis using DeCyder software analyses, protein spots that changed significantly ($p < 0.05$) were selected from the corresponding 2-D DIGE silver stained gels and identified using LC-MS/MS. Results are presented as means $\pm$ SD average ratio. The average ratio represents the relative increase or decrease in protein expression of proteins in LQT1 or LQT2 rabbit hearts as compared to LMC. The results of the proteomic analysis from three independent experiments were eventually analyzed (manually) to identify the most consistently up-regulated or down-regulated proteins in LQT1 and LQT2 as compared to the LMC heart samples.

3. Results

In this study, we employed a fluorescence-based 2-D DIGE proteomic approach to elucidate the differential protein expression in LQT1 and LQT2 as compared to LMC rabbit hearts. The quantitative analysis using DeCyder software (GE Healthcare) enabled us to identify the protein spots that changed significantly ($p < 0.05$). It is evident from the total proteomic analyses that there is differential proteins expression in LQT1 and LQT2 as compared to the LMC hearts (Fig.1). Based on the quantitative analysis using DeCyder software (GE Healthcare), protein spots that changed significantly ($p < 0.05$) were picked up from corresponding 2-D DIGE silver-stained gels. On an average 60-80 spots in each experiment were significantly differentially expressed ($p < 0.05$) and on an average 50-60 spots exhibiting significant changes were picked up from each experiment, and 70-80% of the spots were identified by mass spectrometry with confidence. The proteins that showed consistent changes in expression at least in 2 out of 3 LQT1 versus LMC and LQT2 versus LMC rabbit hearts were considered for studies. Our results using silver-stained gels (Figures 2 and 3) depict the corresponding statistically -significant differential expression in LQT1 and LQT2 rabbit hearts versus LMC corresponding to 2 D DIGE analysis. Overall 18 proteins were identified as differentially expressed from both the LQT1 group (16 upregulated and 2 down regulated, Table 1) and the LQT2 group (17 upregulated and 1 downregulated, Table 2), as compared to LMC hearts. Notably, the proteomic analysis revealed upregulation of key metabolic enzymes involved in all pathways associated with ATP generation.

3.1 Expressions of ATP-generating enzymes are upregulated in LQT1 and LQT2 rabbit hearts

Our proteomic results revealed that most of the identified differentially expressed proteins belonged to enzymes involved in various pathways of ATP generation (Table 1 and 2). Glycogen phosphorylase, which catalyzes the rate-limiting step in the degradation of glycogen by releasing glucose-1-phosphate, eventually providing the substrate for glycolysis, was upregulated 1.3- and 2.16- fold in LQT1 and LQT2 hearts, respectively. There are three major energy-producing processes related to carbohydrate metabolism: glycolysis, the citric acid cycle, and the electron transport chain. It is evident from the Tables 1 and 2 that the expression level of several metabolic enzymes involved in energy producing reactions of carbohydrate metabolism are upregulated in both LQT1 and LQT2 hearts as compared to LMC. For example, glycolytic enzymes upregulated in LQT1 and LQT2 hearts include fructose-1,6-bisphosphate aldolase (1.29- and 1.27-fold, respectively), beta-enolase (2.14- and 1.32-fold), pyruvate kinase (1.21- and 1.6-fold), and L-lactate dehydrogenase beta chain (1.82-fold in both LQT1 and LQT2). Additionally, pyruvate dehydrogenase E1 complex was upregulated in LQT1 and LQT2 hearts (1.26- and 1.96-fold). With regard to the citric acid cycle, we observed upregulation of ATP-specific succinyl-CoA synthetase beta subunit (1.22- and 2.53-fold), succinate dehydrogenase (2.12- and 1.64-fold), and malate dehydrogenase (1.76- and 1.29-fold). We also saw upregulation of enzymes involved in the electron transport chain, such as ATP-synthase subunit alpha (1.35- and 1.32-fold), ATP-synthase subunit beta (1.59- and 1.42-fold), and cytochrome b-c1 complex (4.17- and 1.96-fold). Interestingly, in addition to the upregulation of enzymes involved in carbohydrate metabolism, our results also revealed the upregulation of an enzyme involved in β -oxidation of fatty acids, i.e., very long-chain-specific acetyl-CoA dehydrogenase (VLCAD) (1.26- and 1.32-fold). However, our results revealed the upregulation of long-chain specific-acetyl-CoA dehydrogenase in LQT2 rabbit hearts only (1.4-fold). Furthermore, creatine kinase, the key enzyme responsible for the conversion of creatine to phosphocreatine, was also significantly increased in both LQT1 and LQT2 hearts (1.83- and 1.38-fold) as compared to LMC. The results in tables 1 and 2 indicate that most of the proteins which are differentially expressed in LQT1 and LQT2 as compared with LMC rabbit hearts are in fact the same proteins with the exceptions of two proteins: beta enolase and lactate dehydrogenase, which have different observed pIs (7.48 and 4.84 for beta enolase) and (6.11 and 5.85 for lactate dehydrogenase) in LQT1 and LQT2, respectively. We feel that the different isoforms of beta enolase and lactate dehydrogenase might be getting differentially upregulated in LQT1 and LQT2 as compared to the LMC rabbit hearts.

3.2. Activities of the metabolic enzymes are upregulated in LQT1 and LQT2 rabbit hearts

To validate the results of 2-D DIGE proteomic analyses, we measured the enzyme activities of selective enzymes, including glycogen phosphorylase and one enzyme each representing glycolysis (lactate dehydrogenase), pyruvate decarboxylation (total pyruvate dehydrogenase activity), and the citric acid cycle (succinate dehydrogenase). The enzyme assay results in LQT1 and LQT2 as compared to LMC rabbit hearts revealed significant increases in the activities of glycogen phosphorylase (+50%, +65%, respectively Figure 4A), lactate dehydrogenase (+25%, +25%, Figure 4B), and pyruvate dehydrogenase (+31%, +22%,

Figure 4C). However, succinate dehydrogenase activity was significantly increased only in LQT2 as compared to LMC rabbit hearts (+60%, Figure 4D).

To assess mitochondrial function, we measured the activity of the inner mitochondrial membrane enzyme cytochrome c oxidase (complex IV), an enzyme involved in energy production by coupling electron transport with oxidative phosphorylation. The cytochrome c oxidase assay was only performed in LQT1. LQT2 rabbits were unavailable for the current study due to a sudden and an unprecedented loss of the LQT2 rabbit cohorts in our lab. This loss was due to an episode of sudden cardiac death among LQT2 rabbits. The results of the cytochrome c oxidase assay revealed significant increase in the LQT1 as compared to LMC rabbit hearts (+80%, Figure 4E)

3.3. Expression of key enzymes is upregulated in LQT1 and LQT2 rabbit hearts

Since our proteomic results demonstrated the increased expression of several cytosolic as well as mitochondrial enzymes, we undertook western blot analysis to validate these observations by checking the expression of two enzymes, one from cytosolic and one from mitochondrial preparations from LQT1 and LQT2 rabbit hearts. The results of the Western blot analysis (Figure 5A and 5C) revealed that the expression of pyruvate kinase, a key glycolytic enzyme, is increased in LQT1 and LQT2 rabbit hearts as compared to LMC. However, its expression was not increased significantly as compared with the LMC, possibly due to the wide variation of expression in LQT1 and LQT2 rabbit hearts. The expression of the mitochondrial enzyme - (VLCAD), a key enzyme in β -oxidation of fatty acids 12-24 carbons in length, was significantly increased both in LQT1 and LQT2 (+35% and +33%) as compared to the LMC rabbit hearts (Figure 5B and 5D).

4. Discussion

Chemical energy in the form of ATP is crucial for normal heart functions including viability, myocardial pump function, excitation, contraction, relaxation, ion pumping, synthesis and degradation of molecules, molecular trafficking, and systolic and diastolic functions of the heart [42-44]. To meet these requirements, the heart generates ATP by oxidizing a variety of substrates, including fatty acids, glucose, lactate and several amino acids. Although glucose is the primary source of ATP synthesis in the fetal heart, in adult hearts fatty acid oxidation is the primary source of ATP synthesis [43]. However, the adult heart will oxidize any and all available substrates to meet the demand for ATP. For a given physiological environment, the heart switches to the most energy-efficient substrate. Glucose utilization is increased in hypertrophied and failing hearts [45-47], and fatty acid oxidation is decreased [42, 48]. Under various pathophysiological conditions the heart undergoes functional, structural, electrical, and metabolic remodeling. Our studies of the differential protein expression in LQT1 and LQT2 rabbit hearts as compared to LMC hearts employing a proteomic approach is aimed at elucidating the metabolic remodeling that occurs in these hearts.

The results of proteomic analyses demonstrated the increased expression in LQT1 and LQT2 hearts of enzymes in all the major pathways of substrate utilization and ATP generation: glycogenolysis, glycolysis, glucose oxidation, β -oxidation of fatty acid and oxidative phosphorylation in addition to increased expression of creatine-kinase M (Tables 1

and 2). These observations were validated by increased activity of selected enzymes associated with glycogenolysis, glycolysis, the citric acid cycle, and oxidative phosphorylation (Figure 4) as well as Western blot analyses demonstrating increased expression of one of the key enzymes associated with β -oxidation of fatty acid (Figure 5B). Collectively, our results suggest enhanced generation of ATP by glucose and lipid oxidation and via phosphorylation of creatine to phosphocreatine by creatine-kinase M. There is a growing body of evidence suggesting that metabolic activity and arrhythmias are interdependent, as an impaired cellular energetic state not only predisposes to atrial arrhythmias, but atrial rhythm perturbations/abnormalities also influence metabolic activity as demonstrated by recent observations from other groups [20, 30, 31, 33, 49] employing 2-D DIGE technology in conjunction with mass spectrometry and metabolomics studies. This phenomenon very likely holds true in our LQTS models, as our proteomic analyses and the subsequent biochemical analyses reveal the upregulation of metabolic enzymes associated with ATP generation.

These transgenic rabbits have prolonged QT intervals. The prolonged action potential durations result from the elimination of the repolarizing *-IKs* and *IKr* currents [4]. It has been suggested that glycolytic ATP production correlates with the plateau of the ventricular action potential [50], and that action potential duration correlates with intracellular ATP concentrations [51]. Changes in metabolic activity are closely linked to the altered ion channel functions, as metabolic activity has been shown to critically affect the repolarizing K^+ current [52]. It is very likely that the myocytes from LQTS hearts with reduced repolarization reserve and exhibiting prolonged APD undergo metabolic remodeling to compensate for structural and functional remodeling.

Results from our laboratory and from other groups have reported the prolongation of Ca^{2+} transients in LQTS models and human congestive heart failure [53-55], respectively, eventually leading to triggered activity and arrhythmias. Intracellular calcium is essential for muscle contraction and is believed to control the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA). Sarcoplasmic reticulum Ca^{2+} release to the cytosol is exquisitely sensitive to the cellular energy state. Additionally, it has been suggested that cytosolic Ca^{2+} regulates the utilization of ATP by ATPases such as SERCA as well as the mitochondrial production of ATP [56]. Ca^{2+} has been shown to activate several calcium-sensitive mitochondrial dehydrogenases, such as pyruvate dehydrogenase, isocitrate dehydrogenase, and α ketoglutarate dehydrogenase [57-59] in addition to rapidly activating several steps in oxidative phosphorylation, including F_1F_0 ATPase, which synthesizes ATP [56]. This suggests that Ca^{2+} functions as a cytosolic signaling molecule with a critical role in balancing ATPase activity and metabolism [56]. Thus, our findings suggest that prolongation of APD and the prolonged Ca^{2+} transients observed in LQTS models result in metabolic remodeling of the heart to generate an enhanced amount of ATP to meet the elevated energy demands and to maintain the Ca^{2+} concentration in the sarcoplasmic reticulum.

In summary, our results reveal that both LQT1 and LQT2 rabbit hearts exhibit the increased differential expression of the similar ATP generating enzymes as compared with LMC. This differential expression does not apply to the isoforms of two enzymes: beat-enolase and

lactate dehydrogenase which may be differentially upregulated in LQT1 and LQT2 rabbit hearts. Future research is needed to better elucidate whether these enzymes are differentially post-translationally modified proteins. However, our findings are consistent with results from previous research demonstrating the upregulation of creatine kinase-type M, beta-enolase, lactate dehydrogenase in myocardial infarction in mice heart and congestive heart failure in dog hearts [18, 33], malate dehydrogenase in atrial fibrillation [31], E1 component of pyruvate dehydrogenase in chronic heart failure, dilated cardiomyopathy, and congestive heart failure [17, 19, 33], and ATP synthase subunit alpha and ATP synthase subunit beta in chronic heart failure and congestive heart failure models [31, 33]. Furthermore, the results reported herein pertaining to the increased expression of Lamin-A in both LQT1 and LQT2, as well as of desmoplakin in LQT2 rabbit hearts, are in agreement with the observations that put forth the involvement of these proteins in arrhythmogenic cardiomyopathies [60, 61].

5. Conclusions

In this study we investigated the differential protein expression in LQT1 and LQT2 rabbit hearts as compared to LMC. The results revealed increases in the expression and activities of key ATP-generating enzymes in LQT1 and LQT2 rabbit hearts. Our results suggest an increased demand for ATP in these transgenic rabbit hearts that results in upregulation of enzymes involved in energy generation across the entire metabolic pathway.

6. Clinical Implications

The changes that we have observed in enzymes involved in ATP generation in LQTS rabbit hearts reflects the abnormal energetic that could lead to increased formation of reactive oxygen species and the oxidative stress upon sympathetic stimulation with isoproterenol or metabolic stress. Indeed, arrhythmias in LQT1 and LQT2 are associated with either sympathetic surge (LQT2) or increased sympathetic tone (LQT1) both of which could lead to oxidative stress, which could eventually lead to abnormal calcium dynamics and calcium oscillations that we have recently reported in LQT2 rabbits [62, 63]. Thus, we feel that the findings reported herein could contribute to trigger activity and malignant arrhythmias in long QT syndrome.

7. Limitations of such 2-D DIGE technology

Although, DIGE is a useful tool in the study of differential protein expression, it has some limitations. In particular, the 2D DIGE approach fails to analyze extremely acidic, basic or hydrophobic proteins such as membrane-bound proteins, and very high and small molecular weight proteins. In order to overcome these limitations, we plan to employ the Isobaric tags for relative and absolute quantitation (iTRAQ) - a non-gel-based technique to complement our 2-D DIGE results and as well as to elucidate the differential expression of protein that we might have missed using the 2-D DIGE approach.

Results from our lab have demonstrated the increased calcium load/prolonged Ca^{2+} transient in these transgenic rabbit hearts. Based on these previous observations, we expected to find changes in calcium handling proteins in our LQTs models. Surprisingly, our proteomic analyses results did not reveal any such changes in calcium handling proteins in the

transgenic rabbit hearts. This discrepancy may be attributed to the sensitivity of the 2-D DIGE system as well as to the criteria of significant changes (i.e. 20% upregulation or downregulation) that we undertook to demonstrate the relative differential expression of proteins in LQT1 and LQT2 as compared to LMC rabbit hearts. In addition, it is likely that the changes in the expression of calcium handling proteins/ion channels were below the level of 20% changes and thus could not be observed.

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Highlights

- 2-D DIGE was used to analyze the proteomes in long QT syndrome I and II rabbit hearts
- Differentially expressed proteins were identified in long QTS rabbit hearts
- There was increased expression of enzymes involved in ATP generation
- Enzymatic assays and western blot analyses validated 2-D DIGE LC-MS/MS results

Fig. 1.

Representative 2D DIGE gels of proteins differentially expressed in LQT1 and LQT2 rabbit hearts. The cellular proteomes of LQT1 and LQT2 were compared with LMC heart using DIGE. Proteins from LQT1 (A) and LQT2 (C) were labeled with Cy5 and from LMC labeled with Cy3, whereas, in converse experiment proteins from LQT1 (B) and LQT2 (D) were labeled with Cy3 and those from LMC were labeled with Cy5.

Fig. 2. Representative silver-stained 2D-gel of LQT1 heart proteins. Total protein extracts (150 μ g) from LV of LQT1 and LMC were separated on pH 3-10 NL IPG strip followed by 10% SDS PAGE using DIGE technology. Differentially expressed proteins were indicated by DeCyder software. The protein spots showing significant changes were excised from the corresponding silver stain gel as indicated by numbers and identified by mass spectrometry. The corresponding identifications are listed in Table 1.

Fig. 3.

Representative silver-stained 2D-gel of LQT2 heart proteins. Total protein extracts (150 μ g) from LV of LQT2 and LMC were separated on pH 3-10 NL IPG strip followed by 10% SDS PAGE using DIGE technology. Differentially expressed proteins were indicated by DeCyder software. The protein spots showing significant changes were excised from the corresponding silver stain gel, as indicated by numbers on the figure and identified by mass spectrometry. The corresponding identifications are listed in Table 2.

Fig. 4.

Activities of metabolic enzymes in LQT1 and LQT2 as compared to the LMC rabbit hearts. **A**, glycogen phosphorylase. **B**, lactate dehydrogenase. **C**, pyruvate dehydrogenase, and **D**, succinate dehydrogenase. Enzyme activities were determined as described in the Materials and Methods. The results are shown as mean $\pm$ SD from 3 independent experiments from 3 different LMC, LQT1, and LQT2 rabbits. **E**, cytochrome c oxidase in LQT1 as compared to LMC rabbit heart. Enzyme activity was determined as described in Materials and Methods. The results are shown as mean $\pm$ SD from 3 independent experiments from 2 different LMC and LQT1 rabbits. The asterisks indicate significant differences (* $p < 0.05$), (** $p < 0.01$) and n.s. = non-significant.

Fig. 5.

A, Western blot analysis, **A**, Western blot analysis of pyruvate kinase (20 μ g cytosolic protein per sample) and **B**, Vary long chain-specific acyl Coenzyme A dehydrogenase (VLCAD, 20 μ g mitochondrial protein per sample). Bar graphs indicate the expression levels of pyruvate kinase (**C**), VLCAD (**D**) in 3 different rabbits per group in arbitrary units. GAPDH and UCP2 represent the loading controls. The results are shown as mean $\pm$ SD from 3 different LMC, LQT1, and LQT2 rabbits. The asterisks indicate significant differences ($p < 0.05$).

Table 1
Differentially expressed proteins in LQT1 as compared to LMC rabbit hearts

Spot ID	Protein Name	Protein Accession Numbers	Theoretical pI/MW (kDa)	Observed pLMW (kDa)	Number of Unique Peptides	Percentage Sequence Covered	* Average Ratio	p value
Glucose Metabolism								
1	Glycogen phosphorylase	P11217.6	6.77/97.3	7.52/110.2	3	3.21	1.33	0.0058
18	Fructose-1,6-bisphosphate aldolase	P04075	8.31/39.3	8.54/36.5	4	5.2	1.29	0.0058
13	Beta-Enolase	P13929	7.62/47.0	7.48/49.4	4	7.83	2.18	0.03
6	Pyruvate Kinase; Isozyme M2	P14618	7.61/58.0	8.25/62.7	12	24.11	1.21	0.0059
16	Lactate dehydrogenase	P07195	6.01/36.5	6.11/35.5	7	16.17	1.82	0.001
14	Pyruvate dehydrogenase E1 component	P08559	8.54/43.5	7.88/47.3	2	5.64	1.26	0.0047
12	ATP-specific succinyl-CoA synthetase beta subunit	Q9P2R7	7.57/50.2	6.10/47.5	9	20.42	1.22	0.0015
3	Succinate dehydrogenase	P31040	7.08/72.7	6.33/78.4	3	4.66	2.12	0.0014
17	Malate dehydrogenase, mitochondrial	P04636	8.88/35.5	7.12/34.7	4	15.38	1.76	0.0029
7	ATP synthase alpha subunit, mitochondrial	P19483	9.15/59.7	8.8/70.5	6	12.66	1.35	0.019
9	ATP synthase beta subunit, mitochondrial	P00829	5.15/56.3	4.55/54.5	9	35.57	1.59	0.0081
10	Cytochrome b-C1 complex subunit-1	P31930	5.94/52.6	5.55/50.0	2	5.83	4.17	0.00028
Lipid Metabolism								
5	Very long-chain specific acyl-CoA dehydrogenase	P45953	8.9/70.6	7.22/78.5	4	7.38	1.26	0.0014
15	Creatine kinase-M type	P07310	6.36/43.1	7.45/46.4	5	16.27	1.83	0.043
4	Lamin-A	P02545	6.44/74.0	8.15/88.6	12	17.38	1.37	0.0078
11	Aldehyde dehydrogenase, mitochondrial	P05091	6.68/60.6	6.34/56.7	2	4.26	4.64	0.0015
8	Protein disulfide-isomerase A3 precursor	P11598	5.98/56.3	6.47/58.5	2	4.76	-1.28	0.0065
2	Dynamin-like 120 kDa protein mitochondrial	P58281	7.87/111.6	7.32/86.4	3	3.54	-1.23	0.003

Protein accession number = unique identifier of proteins within Swiss-Prot database. MW = molecular weight; pI = isoelectric pH. P-values are derived from t-test

* A negative or positive average ratio represents a relative decrease or an increase in protein expression of proteins in LQT1 rabbit hearts as compared to LMC, respectively

Differentially expressed proteins in LQT2 as compared to LMC rabbit hearts

Table 2

Spot ID	Protein Name	Protein Accession Numbers	Theoretical pI/MW (kDa)	Observed pI/MW (kDa)	Number of Unique Peptides	Percentage Sequence Covered	* Average ratio	p value
Glucose Metabolism								
1	Glycogen phosphorylase	P11217.5	6.77/97.3	7.15/108.8	4	4.23	2.15	7.00E-05
18	Fructose-1,6-bisphosphate aldolase	P04075	8.31/39.3	8.43/38.4	4	9.07	1.27	0.0046
13	Beta Enolase	P13929	7.62/47.0	4.84/45.9	4	7.4	1.32	0.00027
6	Pyruvate Kinase, Isozyme M2	P14618	7.61/58.0	8.28/64.7	5	8	1.6	0.0059
16	lactate dehydrogenase	P07195	6.01/36.5	5.85/35.5	8	17.07	1.82	0.0041
14	Pyruvate dehydrogenase E 1 component	P08559	8.54/43.5	7.94/49.4	5	11.28	1.96	0.012
12	ATP-specific succinyl-CoA synthetase beta subunit	Q9P2R7	7.57/50.2	6.35/47.5	6	12.91	2.53	0.0005
3	Succinate dehydrogenase	P31040	7.08/72.7	6.44/77.6	3	3.91	1.64	0.00014
17	Malate dehydrogenase, mitochondrial	P04636	8.88/35.5	7.15/37.8	4	15.38	1.29	0.041
7	ATP synthase alpha subunit, mitochondrial	P19483	9.15/59.7	8.74/71.4	12	21.24	1.32	0.00027
9	ATP synthase beta subunit, mitochondrial	P00829	5.15/56.3	4.56/58.4	5	17.65	1.42	0.0013
10	Cytochrome b-Cl complex subunit-1	P31930	5.94/52.6	5.5/56.5	2	3.31	1.96	0.012
5	Very long-chain specific acyl-CoA dehydrogenase	P45953	8.9/70.6	7.3/76.4	4	9.16	1.32	0.0074
11	long-chain specific acyl-CoA dehydrogenase	P15650.1	7.68/47.6	7.4/45.3	5	14.65	1.4	0.013
15	Others	P07310	6.36/43.1	7.62/46.4	6	18.11	1.38	0.018
4	Creatine kinase-M type	P02545	6.44/74.0	7.9/88.6	4	6.27	1.32	0.0074
8	Lancin-A	P15924	7.98/35.0	8.3/38.7	7	2.19	2.62	4.60E-05
2	Desinoplakin	P35609	5.39/104.0	4.6/120.3	4	4.36	-1.29	0.036
	Alpha-actinin 2							

Protein accession number = unique identifier of proteins within Swiss-Prot database. MW = molecular weight; pi = isoelectric pH. P-values are derived from t-test.

* A negative or positive average ratio represents a relative decrease or an increase in protein expression of proteins in LQT2 rabbit hearts as compared to LMC, respectively.