Key Protein Alterations Associated with Hyperdynamic Cardiac Function: Insights Based on Proteomic Analysis of the Protein Phosphatase 1 Inhibitor-1 Overexpressing Hearts

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Transgenesis based on organ specific gene expression has provided the basis to elucidate the functional role of proteins for the past 15 years. Using this technology, we showed that inhibition of the protein phosphatase 1, by its constitutively active inhibitor-1, significantly increases cardiac contractility and calcium handling. To uncover protein changes accompanying the chronic increases in cardiac function of these transgenic hearts, we analyzed the cardiac proteome. Interestingly, we found significant increases in the levels of 6 proteins involved in metabolism, calcium binding and scavenging of oxidoreductive stress. These proteins were identified as: hydroxyacyl CoA dehydrogenase II, alpha subunit of the mitochondrial proton ATPase, peroxiredoxin 2, a novel EF-hand containing protein-2, annexin 5, and a previously uncharacterized cDNA. Thus, long-term cardiac specific overexpression of the protein phosphatase 1 inhibitor-1 and the associated increases in cardiac contractility appear to herald changes in a rather small number of proteins, which may reflect important compensatory adaptations in a hyperdynamic heart.

Rversible protein phosphorylation and dephosphorylation mediated by cellular protein kinases and phosphatases represents one of the most elegant molecular regulatory mechanisms in nature,1 responsible for control of processes as diverse as glycolysis, cell-cycle control and cardiac function. In times of stress, adrenergic signaling is activated throughout the body. In the heart, this leads to increased phosphorylation of various phosphoproteins, such as phospholamban, the L-type calcium channel, the ryanodine receptor, and troponin I.2 These phosphorylation events coordinate the increase of several indices of cardiac contractility, including cardiac inotropy and lusitropy.

Among these cardiac phosphoproteins, phospholamban is a key determinant of sarcoplasmic reticulum calcium cycling and cardiomyocyte relaxation. This small, 52-amino-acid protein is subject to reversible phosphorylation by protein kinase A and dephosphorylation by protein phosphatase 1.2,3 In its dephosphorylated state, phospholamban reduces the calcium affinity of the cardiac sarcoplasmic reticulum ATPase (SERCA). Increased adrenergic drive activates protein kinase A, which phosphorylates phospholamban, resulting in decreased inhibition of SERCA.4 This results in enhanced sarcoplasmic reticulum calcium cycling, improved relaxation and augmented contractility.5 When the protein kinase A activity decreases, protein phosphatase 1 dephosphorylation of phospholamban predominates, restoring phospholamban to its inhibitory, dephosphorylated form.3
In failing hearts, the normal function of the adrenergic signaling axis is compromised and the activity of the protein phosphatase 1 is increased. This results in an overall decreased level of phospholamban phosphorylation, contributing to reduced sarcoplasmic reticulum calcium uptake and diastolic calcium overload, which are characteristic features of heart failure.

In addition, decreased sarcoplasmic reticulum calcium cycling causes a gradual depletion of sarcoplasmic reticulum calcium stores, ultimately resulting in decreased levels of systolic calcium release. Interestingly, augmentation of sarcoplasmic reticulum calcium cycling by inhibition or ablation of phospholamban has shown significant promise in reversing or attenuating heart failure progression. A key approach in reducing phospholamban activity includes inhibition of the phospholamban phosphatase, which results in higher calcium cycling and contractility.

To investigate the physiological and pathophysiological relevance of inhibiting the phospholamban phosphatase in the normal and failing heart, several lines of studies were carried out. Specifically, a constitutively active form of the inhibitor-1 protein, a potent and specific inhibitor of the protein phosphatase 1, was expressed in normal and failing hearts using transgenesis and adenoviral gene transfer technologies. Cardiac-specific expression of this inhibitor protein was associated with increases in cardiac contractility, linked to increased phosphorylation of phospholamban. Interestingly, this strategy was not associated with any changes in the phosphorylation or activity of other cardiac phosphoproteins, including troponin I, the ryanodine receptor, or the L-type calcium channel. Parallel studies in transgenic mice and gene delivery studies in a rat model of heart failure yielded similar results with respect to phospholamban and phosphoprotein phosphorylation, suggesting that the effects of inhibitor-1 were predominantly linked to inhibition of phospholamban dephosphorylation. Based on these findings, it was suggested that inhibitor-1 may constitute a new therapeutic target to augment cardiac function in the failing myocardium.

However, it was important to investigate whether long-term expression of the inhibitor-1 protein, and the associated increased contractility, gave rise to significant changes in the expression of other cardiac proteins, which could contribute to or compromise the enhanced function. While the transgenic methodology is a useful tool in investigating the functional role of proteins, chronic elevation in the levels of a protein is often associated with many secondary adaptations, including compensatory increases and/or decreases in several proteins. Previous proteomic studies in a hyperdynamic model of cardiac function, linked to genetic ablation of phospholamban, revealed increased expression of proteins that regulate energy production, myofilament dynamics, and stress-response pathways.

In this study, the powerful and unbiased proteomic methodology was implemented to uncover compensatory changes associated with long term expression of the protein phosphatase 1 inhibitor-1. Herein, we present the identification of key protein alterations in metabolic and oxido-reductive pathways in this model of hyperdynamic cardiac function, and provide valuable insights into alterations associated with inhibitor-1 therapy in heart failure.

**Materials and Methods**

**Sample preparation**

Hearts were excised from 3 wild-type and 3 constitutively active inhibitor-1 (I-1c) expressing male mice at 3-4 months of age. Connective tissue was trimmed, and the whole heart was finely powdered, using a liquid nitrogen cooled mortar and pestle. The homogenization buffer consisted of 9.5 M urea, 2% CHAPS, 1% DTT, and 0.8% Pharmalyte pH 3-10. This extract was centrifuged for 1 hour at 10,000 g and 4°C. Aliquots of the extract were saved for calculation of protein concentration.

**Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis was carried out at the University of Cincinnati Proteomics Core, as previously described. The gel was removed from the staining tray and placed onto the imaging tray of the Investigator ProImage CCD camera (Genomic Solutions) for image acquisition (multiple exposures). Images were saved as TIFF files for export into the image analysis software. Image analysis, including evaluation of statistical significance, was completed using HT Analyzer v3.1 (Genomic Solutions/Non-Linear Dynamics).

**In-gel digestion of proteins for mass spectrometry**

The protein-acrylamide complex was de-stained and then dehydrated with 25 μL of 1:1 50 mM ammonium bicarbonate: acetonitrile for 10 minutes. Dehydrated gel pieces were treated with dithiothreitol and iodoacetic acid to reduce and alkylate the cysteine. The gel was washed 2 times with 50 mM ammonium bicarbonate and
then dried in a vacuum centrifuge. Following this, digestion with 20 ng/µl trypsin (10 µl) was carried out overnight. Peptides were extracted using 5% formic acid.

**Matrix-assisted laser desorption/ionization time-of-flight analysis**

Matrix-assisted laser desorption/ionization time of flight analysis (MALDI-TOF) was carried out by the Mass Spectroscopy Core at the University of Cincinnati, as previously described. The resulting spectra from MALDI-MS were used to search the SWISSPROT and NCBI databases with the MASCOT algorithm.

**MASCOT analysis**

For each excised protein, a set of unique trypsinized protein fragments exhibited a particular set of mass-to-charge ratios. Based on these ratios, a numerical value was assigned for the predicted protein. This was based on the similarity of the observed mass-to-charge ratios with the predicted ratios for each protein; a score greater than 46 indicated a statistically significant probability of correct protein identification (p<0.05). Only positively identified proteins that met this statistical criterion were included in this manuscript. Six out of eight protein spots excised were positively identified in this way.

**Results**

**Proteomic analysis of active inhibitor-1 expressing hearts**

Hearts were excised from three individual wild-type and active inhibitor-1 expressing male mice and processed for two dimensional proteomic analysis, using a pH 3 to 10 range isoelectric focusing strip, followed by immediate polyacrylamide gel electrophoresis. Upon completion and staining of the gels, a comparison was carried out between the wild-type and transgenic proteome. Using image analysis software, 321 prominent protein spots were matched between wild-type and inhibitor-1 hearts. Statistically significant changes were found in 28 of the 321 protein spots which were compared (p<0.05). Inhibitor-1 expressing hearts exhibited increased expression of 12 proteins and decreased expression of 16 proteins, compared to isogenic, age- and sex-matched wild-type cohort hearts. Of the 12 proteins increased in the inhibitor-1 hearts, 8 proteins were processed for mass spectroscopic analysis, based on protein yield from the corresponding spots on 2D gels.

**Identification of proteins with increased expression in the protein phosphatase 1 inhibitor-1 hearts**

On MASCOT analysis of protein fragments, 6 out of the 8 proteins processed were positively identified, at a level of statistical significance based on mass/charge ratio of trypsinated fragments (Figure 1, Table 1).

Increased expression of several metabolic proteins was observed. Spot #125, which was increased by 11.6 fold in the transgenic inhibitor-1 hearts, was identified as hydroxacyl CoA dehydrogenase II (Hadh2). This protein is alternatively known as SCHAD, a mitochondrial protein and member of the short-chain dehydrogenase family of proteins, responsible for oxidation of free fatty acids. The empirically determined isoelectric
This discrepancy may also be attributed to post translational modifications of this protein, which may include phosphorylation. Fatty acid oxidation is the predominant source of energy in the normal, non-failing heart; upregulation of a key enzyme involved in this process may indicate an important compensatory adaptation in the inhibitor-1 heart. It is likely that increased cardiac function in these hearts was associated with upregulation of the metabolic machinery, illustrating the close link between cardiac function and energetics.

Next, spot #163 was identified as the mitochondrial H+-ATPase alpha subunit (Atp5a1). Interestingly, the actual and calculated isoelectric points again differed markedly for this protein (actual: 6.89 versus predicted of 9.22), while the actual and predicted molecular weights were similar. The more acidic isoelectric point of the mitochondrial ATPase subunit identified may also represent phosphorylation of this protein. The Atp5a1 mitochondrial ATPase alpha subunit represents an important, enzymatically active member of the F0-F1 mitochondrial ATPase complex, which is the final electron acceptor in the mitochondrial electron transport chain, allowing for the generation of ATP, a critical energy source in the heart. The parallel increase in key enzymes responsible for fatty acid oxidation and ATP synthesis in this model of hyperdynamic cardiac function further illustrates the energetic adaptations in hearts with increased contractility.

Significant differences were also uncovered in proteins involved in calcium binding and reduction of oxidative stress. Specifically, spot #146 (7.03 fold elevated) was identified as peroxiredoxin 2 (Prdx2). The empirical and theoretically determined pI and MW were both similar for this protein. The functional significance of peroxiredoxin 2 in the heart has not been previously explored, though current literature supports that the peroxiredoxin family of proteins plays an important role in preventing free radical damage.29,30

The proteomic analysis also revealed a significant increase in other, less well characterized proteins. The level of the EF-hand containing protein 2 (Efhd2), known as swiprosin-1 (spot #103), was elevated 8.2 fold in the active inhibitor-1 expressing hearts relative to the wild-type control. The actual pI/MW of this protein was 4.56/30.7 versus the predicted 5.72/35.9. One of the limitations of proteomic analysis is that this method is more likely to uncover differences in highly expressed proteins. Thus, it is likely that this protein is highly expressed in the heart and may represent an important calcium binding player. Other protein changes identified included annexin A5 (spot #100), which was increased 3.24 fold in the active inhibitor-1 expressing hearts. In addition, the levels of the uncharacterized cDNA 2310005O14Rik were increased 3.46 fold in the active inhibitor-1 overexpressing hearts relative to the wild-type cohorts (spot #102).
Discussion

In an effort to better understand the molecular changes associated with chronic expression of the protein phosphatase 1 inhibitor-1, an important regulator of cardiac contractility and a promising therapeutic target, we carried out cardiac proteomic analysis, which allows for a whole-scale, unbiased examination of protein changes. In this process, significant increases were uncovered, mainly in proteins involved in cardiac metabolism and reduction of oxidative stress. These protein changes may represent important adaptations in a hyperdynamic heart, and comparison of these findings to the pathological changes in heart failure reveals a unique pattern, which may be clinically important.

Critical alterations in fatty acid oxidation and mitochondrial oxidative reductive machinery

The active inhibitor-1 expressing hearts exhibited significant increases in metabolic enzymes consistent with increased energetic demands and energy production. An 11.6-fold increase was observed in the levels of hydroxyacyl coA dehydrogenase II, an important enzyme in the breakdown of fatty-acid chains into acetyl-coA, which is the predominant metabolic process in the heart. In addition, active inhibitor-1 expressing hearts exhibited a 4.05-fold increase in the levels of the mitochondrial proton ATPase-alpha subunit, consistent with increased metabolic activity. Cardiac metabolism in the normal and failing heart represents an important and emerging field of research. Investigators have shown that heart failure is mainly associated with a decrease in fatty acid oxidation and an increase in glycolysis.31 The non-failing heart derives the majority (70-80%) of its high energetic requirements from fatty acids and the remainder from glycolysis (20-30%). In the failing heart, however, there is a shift towards glycolysis; this shift has often been described as a shift towards the fetal phenotype, where glycolysis plays a more prominent role.32,33 Ultimately, the downregulation of the fatty acid oxidation pathway and increased energetic demands of the failing heart lead to a 25-30% decrease in the cell’s steady state ATP levels32,33 and decreased levels of phosphorylated creatine.34,35

A reduction in overall available energy may be a critical deficit in heart failure, and should be amenable to new therapeutic strategies targeting cardiac metabolism.

In our current murine model of hyperdynamic cardiac function, induced by inhibition of the protein phosphatase 1 inhibitor-1, there was upregulation of critical enzymes in the fatty-acid oxidation and oxidative phosphorylation pathways. Interestingly, previous studies in another hyperdynamic model of cardiac function, generated by phospholamban ablation, yielded similar results.27 Phospholamban null hearts also exhibited significant increases of enzymes involved with fatty acid oxidation, including increases in short chain Acyl-CoA dehydrogenase (ACDS) and medium chain Acyl-CoA dehydrogenase (ACDM) levels.27 Future studies using the inhibitor-1 to benefit the failing heart may utilize state-of-the-art technologies to simultaneously measure cardiac function and metabolism, using 31P NMR spectroscopy,31 and determine whether similar energetic alterations enhancing fatty acid oxidation may also be present in heart failure.

Upregulation of peroxiredoxin-2: a novel cardiac oxidoreductive enzyme

Another key change uncovered in the inhibitor-1 expressing hearts was increased levels of peroxiredoxin 2, which scavenges and reduces hydrogen peroxide. Importantly, the increase in the levels of peroxiredoxin 2 in the heart may be tied to the critical changes in metabolic machinery, discussed above. A major consequence of increased cardiac metabolism is the significant increase in formation of oxidative radicals at the level of the cellular NAD(P)H oxidases in the mitochondrial electron transport chain.36 This mechanism represents a major source of free radical generation in cells.36 Generally, increased levels of free radicals can be damaging to tissue, causing significant lipid peroxidation and protein nitration, ultimately leading to cellular dysfunction. Indeed, transgenic overexpression of peroxiredoxin 3 in the mouse heart was shown to forestall heart failure progression in a model of myocardial infarction-induced heart failure, highlighting the importance of mitochondrial oxidative stress reduction in the progression of heart failure.37 Complementary to this gain-of-function experiment, genetic ablation of peroxiredoxin 6 was shown to increase the susceptibility to ischemia-reperfusion injury.38

Interestingly, proteomic analysis of the hyperdynamic inhibitor-1 expressing and phospholamban knockout hearts indicated parallel increases in the levels of peroxiredoxin 2. This protein was elevated by approximately 7 fold in the inhibitor-1 expressing hearts and...
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approximately 4 fold in the phospholamban ablated hearts. The increase in peroxiredoxin 2 in these models may represent an essential adaptation of the hyperdynamic heart to neutralize oxidative stress generated by increased oxido-reductive activity. This energetic adaptation may also be important in heart failure, where multiple anatomic and molecular factors converge to increase the net energetic demands on the heart.

Additional proteomic findings in the inhibitor-1 hearts

In addition, the active inhibitor-1 expressing hearts exhibited increased levels of calcium binding or buffering proteins. The active inhibitor-1 hearts expressed increased annexin 5 levels. Annexins are calcium and phospholipid binding proteins. Many annexins (A2, A4, A5, A7) are expressed in the heart and are reported to control various aspects of vascular and cardiac function. Interestingly, hyperdynamic phospholamban knockout hearts also exhibited increased expression of annexin 2. The exact role of each annexin in the heart remains to be elucidated. Annexin 5 is predominantly expressed in cardiomyocytes and apoptotic signaling triggers its translocation to the cytosolic face of the sarcolemmal membrane. Assays for apoptosis in the active inhibitor-1 expressing heart (TUNEL assays), however, have revealed no increase of DNA fragmentation, which represents a critical event in programmed cell death. Thus, the functional significance of elevated annexin 5 in the inhibitor-1 expressing heart remains unclear.

Active inhibitor-1 expressing hearts also exhibited an increase (spot 103, 8.2 fold increased) in swiprosin-1, a novel EF-hand containing protein. Little is currently known about the role of swiprosin-1 in the cardiomyocyte. However, bioinformatic analysis indicates that swiprosin-1 contains 2 EF-hand domains, suggesting that it may be a buffer for cations, such as calcium, in the heart. Currently, swiprosin-1 has been identified in human lymphocytes, further investigations may reveal its function in cardiac muscle. Due to the bias of in-gel proteome analysis towards abundant proteins, it is likely that swiprosin-1 represents a highly expressed cation binding protein in the heart. Further investigations are necessary to uncover the role and localization of this protein.

Conclusions and clinical significance

In summary, findings from this study illustrate an interesting protein alteration pattern in the hyperdynamic heart, associated with increased expression of inhibitor-1. The increased cardiac energetic demand resulted in compensatory upregulation of the metabolic machinery involved with fatty acid oxidation. This may have increased the levels of oxidative radicals, leading to upregulation of a key cardiac enzyme (peroxiredoxin 2) responsible for quenching of oxidative stress. Future studies may further investigate the functional significance of increased inhibitor-1 activity in the failing heart and the potential associated increases in fatty acid oxidation and peroxiredoxin 2 activity, which may provide additional benefits to the starved heart.

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References

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