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Novel Proteins Associated with Human Dilated Cardiomyopathy: Selective Reduction in α_{1A} -Adrenergic Receptors and Increased Desensitization Proteins

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Abstract

Therapeutics to treat human heart failure and the identification of proteins associated with heart failure are still limited. We analyzed α_1 -adrenergic receptor subtypes in human heart failure and performed proteomic analysis on more uniform samples to identify novel proteins associated with human heart failure. Six failing hearts with end-stage dilated cardiomyopathy and four non-failing heart controls were subjected to proteomic analysis. Out of 48 identified proteins, 26 proteins were redundant between samples. 10 of these 26 proteins were previously reported to be associated with heart failure. Of the newly identified proteins, we found several muscle proteins and mitochondrial/electron transport proteins, while novel were functionally similar to previous reports. However, we also found novel proteins involved in functional classes such as β -oxidation and G-protein coupled receptor signaling and desensitization not previously associated with heart failure. We also performed radioligand-binding studies on the heart samples and confirmed a large loss of β_1 -adrenergic receptors in end-stage dilated cardiomyopathy but also found a selective decrease in the α_{1A} -adrenergic receptor subtype not previously reported. We have identified new proteins and functional categories associated with end-stage dilated cardiomyopathy. We also report that similar to the previously characterized loss of β_1 -adrenergic receptors in heart failure, there is also a concomitant loss of α_{1A} -adrenergic receptors, which are considered cardioprotective proteins.

Keywords

adrenergic; receptor; alpha1; heart failure; desensitization; proteomics

Introduction

Heart failure (HF) is still one of the leading causes of morbidity in developed countries. The non-familial mechanisms involved in the etiology and progression of HF are still largely unknown but are likely to involve changes in gene and protein expression due to the extensive remodeling of the heart. Dilated cardiomyopathy (DCM) is the most prevalent form of cardiomyopathy not due to ischemia and/or hypertension, accounting for about a

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third of all HF cases (1). While there are previous studies utilizing proteomic analysis in cardiac tissue from patients with HF due to DCM (2–6), the number of identified proteins is still small and inter-study verification is almost negligible. Many of these identified proteins can be classified into three functional classes: cytoskeletal and muscle proteins; mitochondrial and electron transport proteins and those involved in stress responses.

One of the most verified proteins that are affected in human HF is the β -adrenergic receptor (AR) as they are the major players in regulating contractility (for review see 7). β -ARs are composed of three subtypes, the β_1 , β_2 and β_3 -AR, with the β_1 - and β_2 -AR being the major subtypes expressed in the myocyte (8). While total β -ARs are decreased in human HF, only the β_1 -AR subtype is selectively affected (9).

α_1 -ARs are also expressed in the heart and can regulate contractility (10), with the α_{1A} - and α_{1B} -AR subtypes being expressed in the human myocyte (11). However, its role in human heart failure is less defined than the β -ARs and previous studies are not at a consensus (11–15). Many of these studies in the 1980s were also performed before optimal radioligand binding studies were developed for the α_1 -ARs.

Our objective was to revisit the role of α_1 -ARs in the context of DCM specifically and to use a more consistent pool of samples from end-stage HF. Using a highly sensitive radioligand, we found that similar to the β -AR scenario, only α_{1A} -ARs selectively decreased in DCM. Using the decrease in β_1 -AR density as means of verifying end-stage HF, we also performed proteomic analysis and identified many more novel proteins associated with DCM in addition to verifying some previously reported proteins.

Methods

Human hearts

Tissue from left ventricular free wall was obtained from six explanted failing hearts of transplant recipients at the Cleveland Clinic Foundation (CCF) with a diagnosis of dilated cardiomyopathy (DCM). Tissue from four non-failing control hearts was from unmatched organ donors through Life Banc of Northeast Ohio. Heart samples were kept in an ice-cold cardioplegic solution (Sivakumar et al., 2008) and tissues from each heart chamber were separated, frozen in liquid nitrogen, and stored at -80°C . All protocols were approved by the CCF Institutional Review Board and all patients gave informed consent. Clinical characteristics of the patients whose failing hearts were used are shown in Table 1. All non-failing donors had normal left ventricular function and no history of myocardial disease or active infection. The hearts were initially considered for cardiac transplantation but were subsequently deemed unsuitable for transplantation either because of blood type or size incompatibility. The cause of death was either cerebrovascular incidents or motor vehicle accidents.

Membrane preparation and receptor radioligand binding

The protocols used for membrane preparation and radioligand binding are previously described (16). Briefly, hearts were homogenized with a polytron in ice-cold buffer composed of 10 mM HEPES, pH 7.4, 250 mM sucrose, 5 mM EGTA, 0.5 mM dithiothreitol, 10 μM phenylmethylsulfonylfluoride, 64 μM benzamidine, and 720 units/l bacitracin, then homogenized with 20 strokes in a Dounce homogenizer. The homogenate was incubated for 30 min in 0.5 M KCl, homogenized again and centrifuged to remove debris. The supernatant was then centrifuged for 20 min at high speed. The pellet was washed twice, then resuspended in 20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EGTA, 12.5 mM MgCl_2 , 0.5 mM dithiothreitol, 10 μM phenylmethylsulfonylfluoride, 64 μM benzamidine, 720 units/l bacitracin supplemented with 10% glycerol. The protein

concentration of this homogenate was measured using the Bradford assay, then stored at -70°C .

Saturation binding was performed using increasing concentrations of ^{125}I -HEAT (2- $[\beta$ -(4-hydroxy-3- ^{125}I iodophenyl)ethylaminomethyl]-tetralone) and 100 μM phentolamine for non-specific binding to determine total α_1 -AR density. To determine the proportion of α_{1A} and α_{1B} -AR binding sites, we performed saturation binding assays in the presence of 10nM 5-methylurapidil to block the α_{1A} -AR, leaving total specific binding attributed to the α_{1B} -AR.

Saturation binding was also performed using ^{125}I -CYP (cyanopindolol) and 1 μM propranolol for non-specific binding to determine total β -AR density. To determine the proportion of β_1 - and β_2 -ARs, we used 50 μM ICI 118551, a selective β_2 -AR antagonist in the saturation binding studies to determine β_1 -AR density. All incubations included 50 μg of membrane protein, shaken at room temperature for 1 h in binding buffer composed of 20 mM HEPES, pH7.4, 1.4 mM EGTA, and 12.5 mM MgCl_2 in a total volume of 500 μl . Assays were performed in triplicate. Bound and free radioactivity was separated by trapping the membranes on a Whatman GF/C filter (preincubated with 0.3% polyethyleneimine), and washing the filter with binding buffer using a cell harvester. Radioactivity bound to the filter was immediately quantified using a gamma counter.

Protein extraction

Proteins from frozen heart tissues were extracted by directly homogenizing the tissues in the lysis buffer (0.5% SDS, 25mM Tris, 2.5mM MgCl_2) containing protease inhibitor cocktail (Calbiochem) with the tissue homogenizer and incubating at 100°C for 10 min. Protein extracts were centrifuged for 15 minutes at 14000rpm to remove cell debris. Protein concentrations were determined using the Bio-Rad protein assay kit according to manufacture's protocol (Bio-Rad, Hercules, CA). The protein extract containing about 500 μg of protein was mixed with six volume of cold acetone and placed at -20°C overnight to precipitate proteins.

2-D PAGE analysis

Equal amount (500–1000 μg) of protein samples were dissolved in 200 μl solubilization buffer (9M urea, 2M thiourea, 1% DTT, 1% Chaps, 1% ampholytes, 1% Triton) and was actively rehydrated into an immobilized pH-gradient (IPG) strip (11cm, pH 3–10) overnight at 50V in Protean IEF Cell (Bio-Rad). Isoelectric focusing (IEF) was performed on IPG strip in Protean IEF Cell with maximum current of 50 μA per strip for a total of 40000 V-Hrs at 20°C following manufacture's instructions (Bio-Rad). Second dimension separation of proteins was performed in precast 12.5% SDS/polyacrylamide gel (Bio-Rad) and the proteins were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) for one hour. The stained gels were scanned and the protein spots on the gels were compared and analyzed.

Protein identification by mass spectrometry

Gel slices of selected protein spots were cut, washed and destained in two aliquots of 50% ethanol/5% acetic acid for 1 hour each. The gel slices were washed in 0.1 M ammonium bicarbonate, and dehydrated in acetonitrile before reduction with dithiotreitol (DTT) and alkylation with iodoacetamide. After reduction and alkylation, the gel pieces were again washed in 0.1 M ammonium bicarbonate, dehydrated in acetonitrile before drying in a speed-vac (Savant Instruments, Holbrook, NY). The sample was rehydrated and digested in 20ng/ μl trypsin in 50 mM ammonium bicarbonate at room temperature overnight. The peptides generated after digestion were extracted from the polyacrylamide gel slice in two aliquots of 30 μl 50% acetonitrile/5% formic acid. These extracts were combined and

evaporated to less than 20 μ l for LC-MS analysis. Two microliters of the concentrated extract were injected and the peptides were eluted from C18 reverse-phase capillary chromatography column with an increasing acetonitrile/0.05 M acetic acid gradient at a flow rate of 0.2 μ l/min. The digest were analyzed using the data dependent multitask capability of a deca quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA) acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. The data were analyzed using all collisionally induced dissociation (CID) spectra collected in the experiment to search the NCBI non-redundant protein sequence database with the search program TurboSequest (Finnigan) for protein candidates. The interpretation process was aided by additional searches using the programs Sequest and Blast as needed.

Western blotting analysis

Heart extracts were washed in phosphate buffered saline and homogenized in a SDS-based lysis buffer (50 mM Tris, 100 mM DTT, 2% SDS, 10% glycerol). Fresh solutions of proteinase inhibitors (final concentrations in lysis buffer: 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 0.15 μ M aprotinin, 0.5 mM EDTA, 1 μ M leupeptin) were added to the lysis buffer immediately before use. Equal amounts of protein were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was immunoblotted with primary antibodies overnight at 4°C. After removal of blotting solution containing primary antibody, the blot was incubated with an HRP-conjugated secondary antibodies at room temperature for 1 h, and the signal was detected by chemiluminescence (Pierce). Total amounts of protein were normalized to β -actin. Images were scanned and analyzed using Image J software.

Antibodies

The following antibodies were obtained from Santa Cruz Biotechnology: Rhodanese (aka thiosulfate sulfurtransferase) (Cat No. SC-20959 at 1:100); Troponin T (Cat No. SC-8121 at 1:5000), Malate dehydrogenase, cytoplasmic (Cat no. SC-101516 at 1:5000), Brain creatine kinase (Cat No. SC-374072 at 1:100), ANP32A (Cat No. SC-100767 at 1:200), Mitochondrial trifunctional protein (aka HADHA, Cat No. SC-374497 at 1:200), Mitochondrial ATPase (ATP5A, Cat No. SC-49164 at 1:5000). Prostatic binding protein antibody was obtained from EMD Millipore (Cat No. 07-137 at 1:250). Nucleolar protein 3 antibody was obtained from Thermo Scientific (Cat. No. PA1-16860 at 1:500). Calreticulin antibody was obtained from Cell Signaling (Cat. No. #2891 at 1:1000). Apolipoprotein A1 antibody was obtained from Abcam (Cat No. Ab7613 at 1:5000).

Statistical Analysis

All data were analyzed using GraphPad Prism using either ANOVA followed by a Bonferroni post-test when analyzing three or more sets of data or using a student's T-test when analyzed two columns of data. Statistical significance is defined as $p < 0.05$.

Results

Composition of Adrenergic Receptor Subtypes

Since human heart failure has long been associated with loss of β_1 -AR receptors and function (9), we performed radioligand binding studies on all of the AR subtypes that are expressed in the myocyte to confirm the diagnosis and severity of DCM, but also to determine additional changes in other AR subtypes. Consistent with previous reports, we found that the total density of β -ARs was comprised of approximately 73% of the β_1 -AR subtype, which decreased from 72 fmoles/mg to 34 fmoles/mg (46% loss) in our HF samples

with no changes in β_2 -ARs (Fig. 2). Surprisingly, we also found that total α_1 -AR density also decreased in HF and non-failing heart was composed of 62% of the α_{1A} -AR. The α_{1A} -AR subtype exclusively decreased in human HF from 33 fmoles/mg to 15 fmoles/mg (Fig. 2).

2D PAGE

Failing and non-failing hearts were subjected to proteomic analysis (Fig. 1.). We performed 2D electrophoresis of three non-failing control hearts and four failing hearts diagnosed with dilated cardiomyopathy (DCM) (Table 1). A number of spots were visually identified to be novel in failing hearts or changing in intensity when compared with non-failing controls. These spots were then excised and subjected to liquid chromatography and mass spectrometry. A total of 49 spots were analyzed and successful proteins were identified for 48 spots. A total of 26 proteins that were consistently found to change between the comparisons are shown in Table 2.

Many of the proteins found during proteomic analysis were previously associated with human HF and confirmed the sensitivity of our analysis. These previously identified proteins were α B-crystallin (16), cardiac type 2 troponin T (17), mitochondrial malate dehydrogenase (3), acetyl coenzyme A acetyltransferase (6), β -myosin heavy chain 7 (18–19), α 2 actin (20), brain (B-type) creatine kinase (21) and transthyretin (22).

There were also several proteins, while previously associated with heart failure, were only described in the serum of human HF patients and used as diagnostic markers and predictive outcomes (22–23). As low serum levels may suggest higher tissue levels, we now report increasing tissue proteins associated with human heart failure are apolipoprotein A1 and albumin (Table 2).

Western Blot Analysis

Of the proteins discovered we confirmed many of these through western analysis depending upon the availability of specific and highly avid antibodies (Table 2). For western blot, we analyzed three non-failing hearts and six failing hearts and these were inclusive of the hearts used in the initial proteomic analysis.

Of those proteins that were decreasing in HF, we found statistically significant decreases in cardiac type 2 troponin T (Fig. 3), mitochondrial malate dehydrogenase (Fig. 4A), mitochondrial F1 ATP-synthase (Fig. 4B), mitochondrial trifunctional protein (MTP)(Fig. 4C), calreticulin precursor (Fig. 5A), prostatic binding protein (Fig. 5B), and rhodanese (Fig. 5C). Changes in MTP, mitochondrial F1 ATP-synthase, calreticulin, prostatic binding protein and rhodanese are the first reports in human HF.

Of those proteins that were increasing in HF, we found statistically significant increases in acidic nuclear phosphoprotein 32A (aka inhibitor of protein phosphatase 2A, IPP2A)(Fig. 6A), apolipoprotein A1 preprotein (Fig. 6B) and nucleolar protein 3 (Fig. 6C). While we did find increased levels of brain creatine kinase (Fig. 6D), it did not reach statistical significance ($p < 0.06$), but likely would have with increased sample numbers.

Of the proteins whose antibodies were either unavailable or of poor quality that rendered western analysis useless, we report that the beta electron-transfer flavoprotein, cytosolic malate dehydrogenase (MDH1, a separate gene product from mitochondrial version MDH2) and myosin light chain 3 are novel proteins in HF that decrease in abundance. γ 2 actin, clathrin light chain A, complement component 1q, manganese superoxide dismutase and α -tropomyosin are also novel proteins in human HF that increase in abundance.

Discussion

Normal human heart is composed of predominately β_1 -ARs that selectively decrease during HF compared to β_2 -ARs (9). The severity of the loss of β_1 -ARs also correlates with the severity of the HF according to the New York Heart Association functional classifications (stages I–IV) with 50% loss of β_1 -ARs equating to stage IV or end-stage heart failure (24). Our findings on the loss of β -ARs and selectively of the β_1 -AR subtype during human HF concur with previous studies (Fig. 2). However, we also found decreased total α_1 -ARs and the selective loss of α_{1A} -AR density (Fig. 2) contrary to a recent report that also compared the α_1 -AR subtypes in human HF (11). In (11), ^3H -prazosin was used as the radiolabel that has 20 times lower specific activity than I^{125} -HEAT. Lower specific activity leads to higher nonspecific binding and detects lower counts of radioactivity, making calculations of low-density receptors difficult. In addition, one-third of the failing hearts had ischemic cardiomyopathy, while our failing hearts were all diagnosed with DCM, a non-ischemic cardiomyopathy.

While the previous literature on the role of α_1 -ARs in human HF report no changes or increases in total α_1 -AR binding (12–14), some studies utilized patients whose failure was caused by pulmonary hypertension (12), a disease demonstrated to induce increased density of α_1 -ARs in the right ventricle (25–27). In (13), only HF functional classes were studied and binding data were not compared to non-failing hearts. In (14), ^3H -prazosin binding was not detected in crude homogenates but suggested increased α_1 -AR density in idiopathic DCM using enriched sarcolemma membranes, but could only detect ^3H -prazosin binding in less than 50% of the samples even in these purified preparations.

Therefore, α_1 -AR density reports are more variable in human HF due to the radioligand used or experimental conditions, but can also vary on the severity of the HF (15), the etiology and/or the thyroid status of the patient (10). Hypothyroidism is known to increase α_1 -ARs and hyperthyroidism decreases receptor density and the sensitivity to α_1 -AR stimulation (28–29). There is a high prevalence of hypothyroidism in HF patients especially in the elderly (30) or those receiving amiodarone therapy (31). In (15), α_1 -AR density only decreased in patients with severe HF (Stage IV), but not in mild to moderate stages of human HF. All of these factors contribute to changes in α_1 -AR density.

In our studies, we used the high specific activity of I^{125} -HEAT and tissue samples from HF patients with severe DCM (Stage IV) as measured by severe decrease in β_1 -AR density by 46% (15). All but one heart were either on LVAD or had 15% or less ejection fraction with an average age of 51 (Table 1) which all suggest severe HF. Our findings on the selective decrease of the α_{1A} -AR are consistent with the studies utilizing LVAD (32–33). These were the first studies documenting changes of different magnitudes in α_1 -AR density in *paired* myocardial tissue samples from patients whose failing hearts were supported by a continuous-flow pump. Those data indicate that mechanical unloading increased α_1 -AR density using a highly-sensitive BODIPY fluorescent prazosin probe (32) and found the major subtype that up regulates and redistributes from the interstitial to the surface of the myocyte is the α_{1A} -AR (33). The changes in total α_1 -AR and α_{1A} -AR density parallel changes seen in the β -ARs and β_1 -ARs not only in HF but the reversal in receptor density seen in end stage HF patients with LVAD (34–35). Our results are also consistent with the current theory that the α_{1A} -AR or α_1 -AR agonism is cardioprotective (36–37). Modest augmentation of cardiac α_{1A} -AR signaling with a subpressor dose of an α_{1A} -selective agonist can prevent doxorubicin-induced cardiomyopathy in mice (38).

After confirmation of the severity of the DCM through adrenergic analysis with Stage IV HF, we performed 2-D PAGE proteomic analysis to determine if severe HF was associated

with any novel proteins. Of the 26 proteins reported in Table 2, 10 were previously associated with human HF and served as a verification of the accuracy of our results. Among these previously reported HF markers are the muscle proteins troponin T (Fig. 3) (17), α 2 actin (20), β -myosin heavy chain 7 (18–19) and nucleolar protein 3 (Fig. 6C)(39). Nucleolar protein 3, also designated ARC for Apoptosis Repressor with Caspase Activation and Recruitment Domains is associated with cardioprotection (39). However, this study showed decreased levels in human HF and we show western blots indicating increased levels (Fig. 6C). Upon inspection of the samples in (39), they were mixed in etiology for HF with no clinical data of age or severity.

Due to its high specificity in heart tissue, serum concentrations of troponin T are well-established diagnostic and prognostic markers of myocardial damage (40). However, novel muscle proteins now identified include myosin light chain 3, γ 2 actin and α -tropomyosin 1 (Table 2). While myosin light chain 2 is known to increase in DCM (41) and increased β -myosin heavy chain 7 contributes to depressed systolic function in HF (18–19), myosin light chain 3 (aka essential ventricular myosin light chain 1) has only been associated with autosomal dominant hypertrophic cardiomyopathic mutations (42). The atrial-specific myosin light chain, a distinct gene, has also been shown to increase expression in human HF (43), but there are no reports to date of myosin light chain 3 expression as a marker for HF. In a similar manner, α -tropomyosin has been associated only with familial DCM (44) and hypertrophic cardiomyopathy (45) and there are no reports of altered expression in HF. In transgenic mice or transfected myocytes, γ 2 actin decreased calcium sensitivity, disassembled myofibrillar thin filaments and inhibited contractility (46–47), so its potential role in human DCM is plausible.

Of the remaining newly identified proteins, 6 are mitochondrial and/or electron transport proteins novel to human HF (Decreasing: Malate Dehydrogenase (Fig. 4A), Mitochondrial F1 ATP-Synthase (Fig. 4B), Electron-Transfer-Flavoprotein Beta, Thiosulfate Sulfurtransferase (Rhodanese)(Fig. 5C); Increasing: Manganese Superoxide Dismutase and Mitochondrial Trifunctional Protein (Fig. 4C)). During the progression of HF, the heart undergoes extensive metabolic remodeling and the failing heart is “fuel deficient” (48) because ATP levels are approximately 30% lower than normal and the rate of ATP loss accelerates. Mitochondria from failing hearts produce more reactive oxygen species (ROS) than normal mitochondria, indicating an association between mitochondrial dysfunction and oxidative stress (49). However, the majority of previous investigations into potential targets responsible for mitochondrial defects in DCM have focused on alterations in the activities and composition of various electron transport chain components in HF (50) as these are the primary source for ROS. In this regard, manganese-containing superoxide dismutase, located in the matrix, eliminates superoxide radical and this enzyme serves to minimize the damaging effects of oxidative free radicals, particularly in the heart (51) and we found that this enzyme increases in human HF (Table 2).

The findings presented in our study are consistent with the theory of cardiac mitochondrial dysfunction in human HF. While several mitochondrial and electron transport proteins are decreasing (Table 2), consistent with lower energy output in HF, we did find novel decreased levels of mitochondrial trifunctional protein (MTP)(Fig. 4C) and electron-transfer flavoprotein, beta. MTP is a multi-enzyme complex regulating β -oxidation of long-chain fatty acids in the generation of ATP (52) and its deficiency would impair energy generation in the heart as the oxidation of fats is the major fuel source. The relative contribution of fats diminishes with enhanced reliance on glucose utilization during the development of HF (53). MTP deficiency is a rare disorder and develops into severe cardiomyopathy in children with particular point mutations (54). However, there is a late-onset and less severe form of MTP deficiency (55). Together with the novel discovery of decreasing electron-transfer

flavoprotein, beta, another enzyme involved in β -oxidation, the recent identification of acetyl coenzyme A acetyltransferase (6), the last enzyme in the pathway that breaks down fat, and the several inherited mutations in other fatty acid metabolism proteins that lead to cardiomyopathies (56) suggests that repression of β -oxidation may contribute to HF progression due to inadequate myocardial energy supply. Interestingly, postmortem analyses of DCM hearts also show accumulation of lipid droplets demonstrating defective β -oxidation (57).

There is accumulating evidence that disturbed calcium homeostasis may play a key role in the pathophysiology of human HF. While calreticulin (Fig. 5A) is essential for cardiac development (58) by regulating intracellular calcium homeostasis and endoplasmic reticulum calcium storage capacity, it is also an important molecular chaperone involved in proper protein folding within secretory pathways. However, its role in HF and pathology is unclear. Calreticulin may be protective in hypertrophy (59) and increased in expression during pressure overload hypertrophy. However, we found that it decreases in human HF (Fig. 5A).

We may also expect several GPCR signaling molecules to be affected in HF given the changes in AR proteins. In novel findings, prostatic binding protein (Fig. 5B) decreases while ANP32A (Fig. 6A) and clathrin both increase in DCM. Prostatic binding protein is a raf kinase inhibitor protein and is a physiological inhibitor of G-protein Receptor Kinase 2 (60), an enzyme which desensitizes and functionally uncouples GPCRs and increases expression in human HF (61). ANP32A is also known as the inhibitor of protein phosphatase 2A (I α PP2A) and increased expression in HF of this protein leads to decreased expression of β -ARs by blocking the dephosphorylation process that resensitizes the receptor back to the cell surface (62). In addition, clathrin-mediated endocytosis is one of the major mechanisms employed by cells for the removal of receptors from the plasma membrane. In human HF, there is increased endocytosis that will decrease surface receptors into early and late endosomes (63). Therefore, human HF is also associated with mechanisms to increase the desensitization and removal of ARs from the cell surface, in accordance with previous binding studies (9).

Our findings suggest a novel therapeutic potential for α_{1A} -AR agonism or resensitization in human HF. Mechanistically, α_{1A} -ARs may follow the exact desensitization scenario of β_1 -ARs in human HF. Therefore, therapeutics that allow the receptor to remain cell surface and/or enhance the function of remaining receptors would be feasible and similar approaches have already been suggested for β_1 -ARs (62–63). The additional benefit of focusing on the α_{1A} -AR is its promising cardioprotective and anti-apoptotic mechanisms through coupling to ERK and STAT3 (36–37, 64–66) in contrast with the pro-apoptosis induced through β_1 -ARs (67–68).

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Figure 1. 2-Dimensional Polyacrylamide Gel Electrophoreses of Failing and Non-Failing Human Hearts

Equal amount of protein samples were dissolved in solubilization buffer and actively rehydrated into an immobilized pH-gradient (IPG) strip (pH 3–10) overnight followed by isoelectric focusing. Second dimension separation of proteins was performed in a 12.5% SDS/polyacrylamide gel and the proteins were stained with Coomassie Brilliant Blue R-250. The stained gels were scanned and the protein spots on the gels were visually compared and removed. A. Representative gel from a non-failing human heart control. B. Representative gel from a failing human heart. Numbers and letters represent some of the spots that were analyzed.

Figure 2. Column Scatter Plot of Adrenergic Receptor Subtype Density in Failing and Non-Failing Human Hearts

The protocols used for membrane preparation and radioligand binding are previously described (16). Saturation binding was performed using increasing concentrations of ^{125}I -HEAT and 100 μM phentolamine for non-specific binding to determine total α_1 -AR density. To determine the proportion of α_{1A} and α_{1B} -AR binding sites, saturation binding assays were performed in the presence of 10nM 5-methylurapidil to block the α_{1A} -AR, leaving total specific binding attributed to the α_{1B} -AR. Saturation binding was also performed using ^{125}I -CYP and 1 μM propranolol for non-specific binding to determine total β -AR density. The proportion of β_1 - and β_2 -ARs was determined by adding 50 μM ICI 118551, a selective β_2 -AR antagonist in the saturation binding studies to determine β_1 -AR density. Horizontal line represents the mean. * $p < 0.05$ compared to non-failing control.

Figure 3. Troponin T Protein Levels in Failing and Non-Failing Human Hearts

A. Western blot analysis was determined as outlined in the methods. Proteins levels were normalized to β -actin. **B.** Bands were analyzed using image J software and the relative density shown. NF, non-failing hearts; F, failing hearts. * $p < 0.05$ compared to non-failing control.

Figure 4. Mitochondrial Protein Levels in Failing and Non-Failing Human Hearts

Western blot analysis was determined as outlined in the methods. Proteins levels were normalized to β -actin. **A.** Mitochondrial Malate Dehydrogenase; **B.** Mitochondrial F1 ATP Synthase; **C.** Mitochondrial Trifunctional Protein. Bands were analyzed using image J software and the relative density shown. NF, non-failing hearts; F, failing hearts. * $p < 0.05$ compared to non-failing control.

Figure 5. Proteins that Decrease in Failing and Non-Failing Human Hearts

Western blot analysis was determined as outlined in the methods. Proteins levels were normalized to β -actin. **A.** Calreticulin; **B.** Prostatic Binding Protein; **C.** Rhodanese. Bands were analyzed using image J software and the relative density shown. NF, non-failing hearts; F, failing hearts. * $p < 0.05$ compared to non-failing control.

Figure 6. Proteins that Increase in Failing and Non-Failing Human Hearts

Western blot analysis was determined as outlined in the methods. Proteins levels were normalized to β -actin. **A.** Acidic Nuclear Phosphoprotein 32A; **B.** Apolipoprotein A1-Preprotein; **C.** Nucleolar protein 3; **D.** Brain Creatine Kinase. Bands were analyzed using image J software and the relative density shown. NF, non-failing hearts; F, failing hearts.

* $p < 0.05$ compared to non-failing control.

Table 1

Characteristics of failing hearts

Age	Sex	Race	Diagnosis	Drugs	Ejection Fraction	LVAD
57	M	White	DCM	DIG, MIL	Unknown	
32	M	White	DCM	DIG, MIL	15	
42	M	White	DCM	AM	Unknown	Yes
63	M	Black	DCM	AM, DIG	10	
49	M	White	DCM	LIS, MET, MIL	Unknown	Yes
62	M	White	DCM	CAPT	Unknown	Yes

AM, AMIODARONE (CORDARONE); CAPT, CAPTOPRIL (CAPOTEN); DCM, DILATED CARDIOMYOPATHY; DIG, DIGOXIN (LANOXIN); LVAD, LEFT VENTRICULAR ASSIST DEVICE; LIS, LISINOPRIL (PRINIVIL, ZESTRIL); MIL, MILRINONE (PRIMACOR); MET, METOPROLOL (LOPRESSOR).

Table 2**Changes in Protein in Human Heart Failure**

Result	Protein	NCBI index #	prior ID	Confirmed WB
Decreasing				
Mitochondrial and/or Electron Transport				
	Mitochondrial Malate Dehydrogenase	21735621	Knecht, 1994	Yes
	Ubiquinol-cytochrome C Reductase Core protein I	46593007	Li, 2012	
	Mitochondrial F1ATP-Synthase	4757810	Novel	Yes
	Electron-transfer-flavoprotein, beta polypeptide isoform	14503609	Novel	
	Thiosulfate Sulfurtransferase (Rhodanese)	17402865	Novel	Yes
	Acetyl Coenzyme A Acetyltransferase I	4557237	Rosello, 2012	
	Cytosolic Malate Dehydrogenase	5174539	Novel	
	Mitochondrial Trifunctional Protein	20127408	Novel	Yes
Muscle				
	Cardiac Type 2 Troponin T	48255877	Hessel, 2008	Yes
	Myosin Light chain 3	4557777	Novel	
Chaperones				
	α B-Crystallin	4503057	Yang, 2000	
	Calreticulin precursor	4757900	Novel	Yes
Other				
	Prostatic binding protein (Raf kinase inhibitor protein)	4505621	Novel	Yes
Increasing				
Muscle				
	Alpha 2 Actin	4501883	Barrans, 2002	
	Beta-Myosin heavy chain7	4557773	Abraham, 2002	
	Gamma 2 Actin	4501889	Novel	
	Tropomyosin 1, alpha chain isoform 1	63252898	Novel	
	Nucleolar protein 3	4505419	Donath, 2006	Yes
Desensitization				
	Acidic Nuclear Phosphoprotein 32 A (IPP2A)	5453880	Novel	Yes
	Clathrin, light polypeptide A	4502899	Novel	
Mitochondrial				
	Manganese Superoxide Dismutase, isoform A, precursor	67782305	Novel	
Blood				
	Apolipoprotein A-I Preprotein	4557321	Novel in tissue	Yes
	Albumin	4502027	Novel in tissue	
Other				
	Brain (B-Type) Creatine Kinase	21536286	Hosseini-Nia, 1997	Yes
	Complement component Iq	4502491	Novel	
	Transthyretin	4507725	Li, 2012	