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RESEARCH ARTICLE

Proteomic analysis of membrane microdomains derived from both failing and non-failing human hearts

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Eukaryotic cells plasma membranes are organized into microdomains of specialized function such as lipid rafts and caveolae, with a specific lipid composition highly enriched in cholesterol and glycosphingolipids. In addition to their role in regulating signal transduction, multiple functions have been proposed, such as anchorage of receptors, trafficking of cholesterol, and regulation of permeability. However, an extensive understanding of their protein composition in human heart, both in failing and non-failing conditions, is not yet available. Membrane microdomains were isolated from left ventricular tissue of both failing ($n = 15$) and non-failing ($n = 15$) human hearts. Protein composition and differential protein expression was explored by comparing series of 2-D maps and subsequent identification by LC-MS/MS analysis. Data indicated that heart membrane microdomains are enriched in chaperones, cytoskeletal-associated proteins, enzymes and protein involved in signal transduction pathway. In addition, differential protein expression profile revealed that 30 proteins were specifically up- or down-regulated in human heart failure membrane microdomains. This study resulted in the identification of human heart membrane microdomain protein composition, which was not previously available. Moreover, it allowed the identification of multiple proteins whose expression is altered in heart failure, thus opening new perspectives to determine which role they may play in this disease.

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Abbreviations: eNOS, endothelial nitric oxide synthase; **FABP**, fatty acid binding protein; **GSTO**, GST omega 1; **HSP**, heat shock protein; **IF**, intermediate filaments; **MyHCs**, myosin heavy chain; **PRDX**, peroxiredoxin; **smHSP**, small HSP; **Tn**, troponin; **VDAC**, voltage-dependent channel

1 Introduction

Heart failure is a severe disease characterized by impaired systolic function with reduced ejection fraction and increased end-systolic blood volume, resulting in dilatation of one or both ventricles as the heart adapts to maintain a normal stroke volume [1]. Heart failure is now widely regarded as a syndrome in which a variety of etiological and

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predisposing factors give rise to myocardial dysfunction, ultimately reflected in a similar pathological condition. A large number of qualitative and quantitative changes in cardiac proteins have been described in biochemistry or pathology studies or proposed on the basis of mRNA expression studies in a combination of animal model and human disease samples.

The cardiac remodeling associated with heart failure includes important modifications of both cellular and subcellular organization, including alterations in levels of G proteins, membrane receptors and calcium regulatory proteins, and contractile protein abnormalities [1].

The results of numerous studies indicate that G protein-coupled receptors and second messenger systems are not randomly distributed in cell membranes, but move within the plasma membrane where they segregate into membrane microdomains [2, 3].

Indeed, in eukaryotic cells plasma membranes are organized into domains of specialized function such as lipid rafts and caveolae. These are membrane microdomains representing subcompartments of the plasma membrane with a specific lipid composition, being highly enriched in both cholesterol and glycosphingolipids [4–6]. In addition to their role in regulating signal transduction, multiple functions have been proposed, such as anchorage of various receptors, trafficking of cholesterol, and regulation of permeability, among others [7–12].

To appreciate the full scope and complexity of membrane microdomains-dependent functions in human heart, it is desirable to have an extensive understanding of their protein composition, which, as yet, is not available. Their low buoyant density provides a simple and effective biochemical approach to the isolation and characterization of membrane microdomain proteins [13]. A combination of improvements over standard proteomics protocols, and rapid advances in the identification of proteins by sequence-based MS techniques allow significantly enhanced separation and identification of proteins present in a wide range of biologically relevant samples. Since membrane microdomains represent a minor and highly selected subset of the cellular proteome, they are particularly well suited to investigation using MS techniques. Using this approach, valuable protein information regarding localization, possible function and potential PTMs has been gathered concerning the composition and functional aspects of human heart membrane microdomains-associated proteins during the development of heart failure.

2 Materials and methods

2.1 Materials

Anti-caveolin monoclonal and polyclonal antibodies and anti-endothelial nitric oxide synthase (eNOS) were purchased from Transduction Laboratories (Lexington, KY, USA); anti-G protein α antibody from Biosource (Camarillo, CA, USA); anti-src antibody from Cell Signaling (Beverly, MA, USA); and anti-β_2-adrenergic receptor antibody from Santa Cruz (Santa Cruz, CA, USA). Secondary antibodies, carrier ampholytes, urea, dithiothreitol (DTT), IPG strips were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals, including thiourea, MES, and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO, USA).

2.2 Heart tissue samples

Non-failing left ventricular tissue (apex) was obtained from 15 donor hearts used for valve harvest at Monzino Cardiologic Center and immediately frozen at −80°C. Seven of the 15 donors were male and the mean age was 44 ± 2.2 years. Tissue from 15 failing hearts (apex) was obtained from patients undergoing cardiac transplantation at Niguarda Hospital. Causes of heart failure included idiopathic dilated cardiomyopathy ($n = 8$) and ischemic cardiomyopathy ($n = 7$). Six of the patients were male and the mean age was 52 ± 5 years. The ejection fraction was <20%. None had been treated with left ventricular assistance devices or had received chronic intravenous inotropic support for at least 7 days before transplantation. Heart failure therapy consisted of angiotensin-converting enzyme inhibitors and diuretics in all patients. The study protocol was approved by the ethics committee of Monzino Cardiologic Center and Niguarda Hospital.

2.3 Membrane microdomains isolation from human heart tissue lysates

Isolation of membrane microdomain fractions was performed following the previously described procedure [14]. Briefly, cardiac tissue was homogenized in 0.5 mol/L Na_2CO_3 pH 11 with protease inhibitors (1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin), with a buffer to tissue ratio of 1 mL buffer to 200 mg tissue. The sample was homogenized at setting 5 of a Polytron homogenizer (three 10-s bursts), and sonicated using a Branson Sonifier 250. After centrifugation at $1000 \times g$, 2 mL of the lysate was adjusted to 45% sucrose by mixing with 2 mL of 90% sucrose prepared in 25 mmol/L MES, pH 6.5, containing 150 mmol/L NaCl, and placed at the bottom of a 5% and 35% discontinuous sucrose gradient (in MES containing 100 mmol/L Na_2CO_3) for an overnight ultracentrifugation in Beckman SW41 rotor at 39 000 rpm. From the top, 1-mL fractions were removed sequentially and designated as fractions 1–12. Protein concentration in each fraction was quantified by Bradford assay [15]. Cholesterol concentration was analyzed using Roche Diagnostics enzymatic kit.

2.4 Gel electrophoresis and immunoblotting analysis

Aliquots of the harvested fractions were resuspended in Laemmli buffer [16] containing 1 mmol/L PMSF, 100 U/mL aprotinin and 5 mmol/L benzamidine. Proteins were electrophoretically separated on a 12% SDS-polyacrylamide gel and transferred to NC membranes, as previously described [17].

2.5 Sample preparation and 2-DE

Samples enriched in membrane proteins require specific adaptations of existing protocols involving sample preparation, spot handling and separation. Because membrane microdomains fractions have specific lipid compositions and are enriched in membrane proteins, several extra steps were included and optimized, regarding delipidation, solubilization, focusing, and recovery of peptides. Aliquots of membrane fractions were concentrated and delipidated by a TCA/acetone procedure. Pelleted membrane protein fractions were first presolubilized in 50 μL 1% SDS, 1% DTT and 20 mmol/L Tris followed by shaking for 1 h. After complete solubilization, buffer was added yielding final concentrations of 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 2% carrier ampholytes pH 3–10, 20 mmol/L Tris, 55 mmol/L DTT, and bromophenol blue. 2-DE was carried out according to the manufacturer's protocol (Protean IEF cell, Bio-Rad). IPG ready strips, 11 cm, pH 3–10 non-linear gradient (Bio-Rad), were actively rehydrated at 50 V for 24 h. Proteins (400 μg) were loaded at the anode using the cup loading tray for Protean IEF cell (Bio-Rad) and focused for a total of 20 kVh. After focusing, the strips were first equilibrated for 15 min with a solution containing 50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 2% SDS and 2% DTT, and then with the same buffer containing 4.5% iodoacetamide instead of DTT. The focused proteins were then separated according to size by SDS-PAGE on 7–17% polyacrylamide gradients (14 × 14 cm), and stained with colloidal blue stain.

2.6 Gel comparison and statistical analysis

To evaluate the reproducibility and to improve the reliability of the qualitative and quantitative changes in protein expression, we analyzed the samples in triplicate. PDQuest software (Bio-Rad) was used for spot detection, spot quantification, gel matching, and statistical analysis of differences between the experimental groups; 330 spots per gel were detected, with a CV below 10% in the 82% of the cases. The cut-off level for a differentially expressed protein was defined as at least a twofold increase or decrease in spot intensity. Statistically significant differences between groups for each protein were computed by ANOVA. The level of significance of difference was set at $p < 0.05$.

2.7 MS analysis

In-gel digestion with trypsin was performed according to published methods [18], modified for use with a robotic digestion system (Genomic Solutions, Huntington, UK). Tandem electrospray mass spectra were recorded using a Q-TOF hybrid quadrupole/orthogonal acceleration TOF spectrometer (Waters, Manchester, UK) interfaced to a Waters CapLC apparatus. Samples were dissolved in 0.1% aqueous formic acid, injected onto a Pepmap C18 column (300 μm × 5 mm; LC Packings, Amsterdam, The Netherlands), and eluted into the electrospray with an ACN/0.1% formic acid gradient (5–70% ACN over 20 min). The capillary voltage was set to 3500 V, and data-dependant MS/MS acquisitions were performed on precursors with charge states of 2, 3 or 4 over a survey mass range 540–1200. The collision gas was argon, and the collision voltage was varied between 18 and 45 V depending on the charge-state and mass of the precursor. Known trypsin autolysis products and keratin-derived precursor ions were automatically excluded. Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in Swiss-Prot/TREMBL, using ProteinLynx Global Server (V 1.0; Micromass) [18]. One missed cleavage per peptide was allowed, and the fragment ion tolerance was set to 100 ppm. Carbamidomethylation of cysteine was assumed, but other potential modifications were not considered in the first pass search. All matching spectra were reviewed manually, and in cases where the score reported by ProteinLynx global server (V 1.0; Micromass) was less than 100, additional searches were performed against the NCBI nr database using MASCOT, which utilizes a robust probabilistic scoring algorithm [19].

3 Results

3.1 Isolation of membrane microdomains from human heart ventricles

Human heart membrane microdomains were separated using the previously described discontinuous sucrose density gradient fractionation method, yielding 12 fractions (numbered 1–12, with sample 1 being the lightest top fraction) with distinct protein compartmentalization [14, 20, 21]. A single light-scattering band corresponding to a low-density complex was observed mainly in fractions 4–5 of the sucrose gradients (at the interface 5–35% sucrose). These fractions were enriched in cholesterol, and excluded ≥90% of the total cellular protein which remained within the bottom-loaded 45% sucrose layer (fractions 8–12) (Fig. 1). A yield of 250 μg, from fraction 4, was obtained from 300 mg of the starting material. An array of proteins (ranging from receptor tyrosine kinases, G protein-coupled receptors, ion channels, adaptor proteins, and structural proteins) has been reported to be preferentially localized to membrane microdomains [12, 22, 23]. Figure 2 illustrates that, in this fractionation

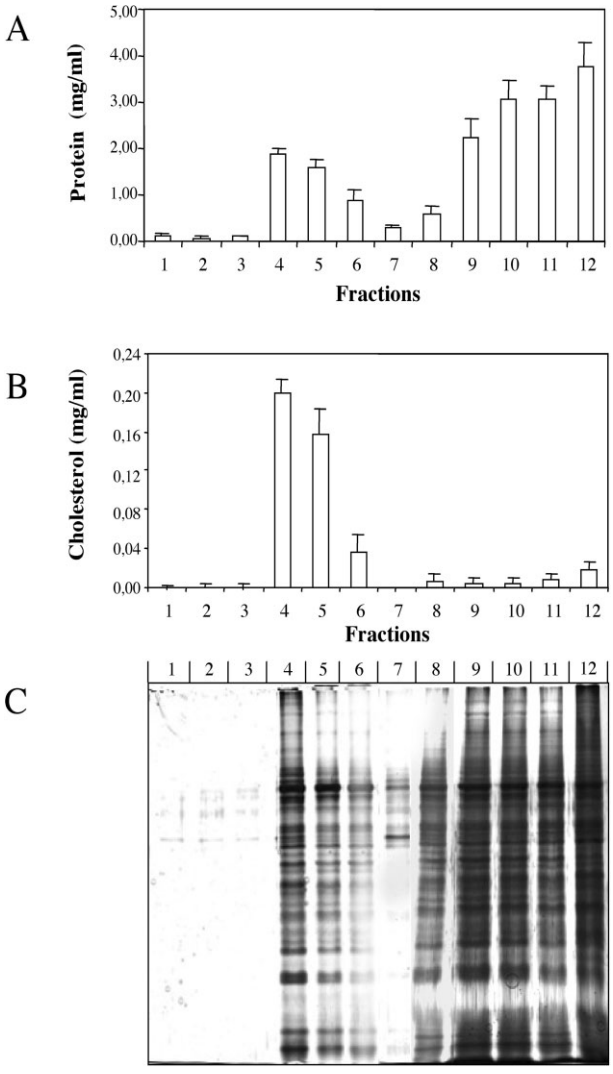

Figure 1. Isolation of membrane microdomains from human heart. Sucrose gradient fractions were collected and analyzed for protein (A) and cholesterol (B) content. (C) SDS-PAGE of each separated fractions.

scheme, caveolin-1 (fraction 4) is separated from the bulk of cellular proteins. In addition, a series of known caveolin-associated proteins, such as G protein subunits, Src-family tyrosine kinases, eNOS, and β_2-adrenoceptor, were co-purified mainly in fraction 4 (Fig. 2). The caveolin-enriched fractions, containing proteins from both caveolae and raft membrane domains were collected and processed for subsequent analysis.

3.2 Characterization of membrane microdomains from human cardiac left ventricular tissue: 1-DE

Recent advances in the ability to resolve complex protein mixtures through a combination of MALDI-TOF and MS/MS, coupled with reports that proteins are incompletely

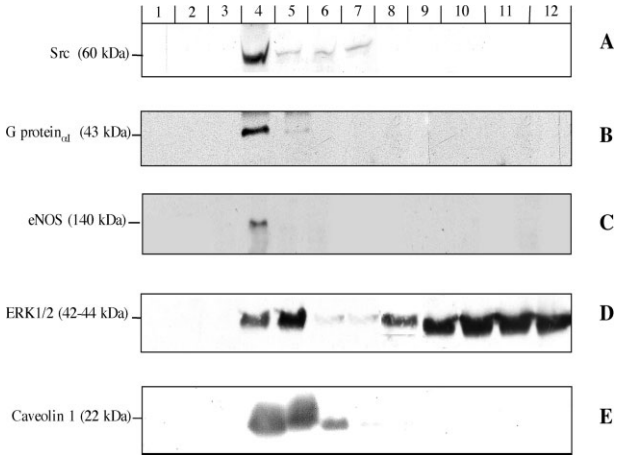

Figure 2. Immunoblotting of proteins co-purifying with human heart membrane microdomains. Sucrose gradient fractions were separated by 12% SDS-PAGE and, after transfer to NC, blotted with anti-eNOS (A), anti-src (B), anti-β_2-adrenergic receptor (C), anti-G protein (D) and anti-caveolin (E) antibodies.

represented in 2-D polyacrylamide gels, prompted us to initially analyze the proteins in one dimension. To identify the major protein components of membrane microdomains, fraction 4 was resolved by SDS-PAGE (Fig. 3). Gel bands were then excised and digested for subsequent MS analysis as described in the Materials and methods section. Table 1 lists all the proteins identified from the bands excised from the gel.

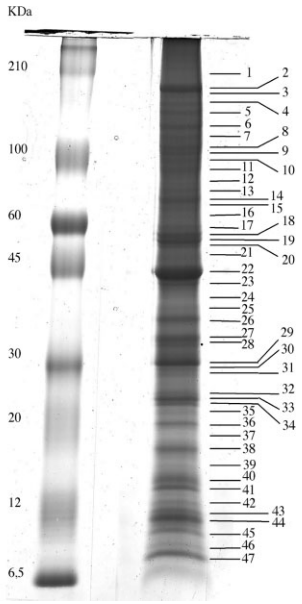

Figure 3. Separation of membrane microdomain proteins on 7–17% gradient SDS-PAGE for identification by MS. Membrane microdomain proteins (200 μg) were run on 7–17% gradient SDS-PAGE and stained by CBB. Numbered bands were cut out from the gel and identified, after trypsin digestion, by LC-MS/MS.

Table 1. Identification of membrane microdomain proteins based on PMF and MS/MS-derived sequences

Name	Accession code[a)]	Mol. mass (kDa)	Sequence coverage %/ MOWSE score	Sequence	Band no.
Acetyl CoA acetyltransferase	THIL_HUMAN	45.2	3.9/246	(R)TPIGSFLGSLSLLPATK(L) (R)QAVLGAGLPISTPCTTINK(V) (K)AAWEAGKFGNEVIPVTVTVK(G)	19
Aconitate hydratase	ACON_HUMAN	85.4	2.1/214	(K)IVYGHLDDPASQEIER(G)	11
Actin, alpha cardiac	ACTC_HUMAN	42.1	10.9/975	(K)CDVDIRK(D) (K)AGFAGDDAPR(A) (R)AVFPSIVGRPR(H) (K)DSYVGDEAQSKR(G) (R)LDLAGRDLTDYLMK(I) (K)SYELPDGQVITIGNER(F) (R)TTGIVMDSGDGVTHTVPIYEGYALPHAIL(R)	20
Alpha-actinin 2	AAC2_HUMAN	103.8	2.46/116	(R)ISNRPAFMPSEG(K)	9
Alpha crystallin B chain	CRAB_HUMAN	20.2	30.3/555	(R)FSVNLDVK(H) (R)EEKPAVTAAPK(K) (R)LFDQFFGEHLLESDLFPTSTSLSPFYL(R)	36
Adenylate kinase 1	KAD1_HUMAN	21.3	9.2/49	(K)GF LIDGYPR(E)	34
ADP, ATP carrier protein	ADT1_HUMAN	33.1	22.5/572	(R)YFPTQALNFAFK(D)	29
Amine oxidase B	AOFB_HUMAN	58.6	2.1/71	(K)FVGGSGQVSER(L) (K)GPFPPVWNPITYLDHNNFW(R)	15
ATP synthase alpha chain	ATPA_HUMAN	59.7	25.1/1018	(K)HALIIYDDLSK(Q) (K)TSIAIDTIINQK(R) (K)LKEIVTNFLAGFEA(R) (R)ILGADTSVDLEETGR(V) (R)TGAIVDVPVGEELLGR(V) (R)VVDALGNAIDGKGPIGSK(T) (R)EVAAFAQFGSDLDAATQQLLS(R) (K)FENAFLSHVVSQHQALLGTIR(A) (R)ILGADTSVDLEETGRVLSIGDGIAR(V)	18
ATP synthase beta chain	ATPB_HUMAN	56.5	13.6/296	(R)FTQAGSEVSALLGR(I) (K)TVLIMELINNVAK(A) (R)LVLEVAQHLGESTVR(T) (K)VLDSGAPIKIPVGPETLGR(I) (K)IGLFGGAGVGKTVLIMELINNVAK(A)	19
ATP synthase D chain	ATPQ_HUMAN	18.3	10.6/597	(R)LAALPENPPAIDWAYYK(A)	34
ATP synthase E chain	ATPJ_HUMAN	7.8	14.7/55	(R)ELAEDDSILK	47
ATP synthase F chain	ATPK_HUMAN	10.7	13.9/191	(R)DFSPSGIFGAFQR(G)	45
ATP synthase coupling factor 6	ATPR_HUMAN	12.6	21.3/332	(R)QTSGGPVDASSEYQQELERELFK(L)	46
Cadherin-13 (heart cadherin)	CADD_HUMAN	78.3	3.2/117	(R)DVG KVVDSDRPER(S)	8
Calcium binding mitochondrial carrier protein	CMC_HUMAN	74.7	5.6/105	(R)QQSPGLGRPIWLQIAESAYR(F) (R)IAPLAEGALPYNLAELQR(Q)	14
Calnexin	CALX_HUMAN	67.6	2.5/88	(R)KIPNPDFFEDLEPFR(M)	9
Caveolin-1	CAV1_HUMAN	20.3	4.5/31	(K)HLNDDVVK(I)	34
CD36 platelet glycoprotein IV	CD36_HUMAN	52.9	3.6/141	(R)TYLDIEPITGFTLQFAK(R)	10
CD59 glycoprotein precursor	CD59_HUMAN	14.1	6.2/61	(K)AGLQVYNK(C)	37
Creatine kinase	KCRS_HUMAN	47.5	4.77/58	(R)EVENVAITALEGLKGDLAGR(Y)	21
Creatine kinase M chain	KCRM_HUMAN	43.1	10.8/240	(R)GIWHNDNK(S) (K)LSVEALNSLTGEFKGK(Y) (R)LGSSEVEQVQLVVDGVK(L)	22

Table 1. Continued

Name	Accession code[a)]	Mol. mass (kDa)	Sequence coverage %/ MOWSE score	Sequence	Band no.
Cytochrome C1	CY1_HUMAN	35.4	8.6/147	(R)GLSSLDHTSIR(R) (R)AANNGALPPDLSYIVR(A)	28
Cytochrome C oxidase subunit IV	CX41_HUMAN	19.6	6.5/269	(R)DHPLPEVAHVK(H)	40
Cytochrome C oxidase subunit V	Q71UP1[b)]	4.2	15.9/71	(R)LVPQQLAH(K) (K)GLDPYNVLAP(K)	43
Cytochrome C oxidase subunit VI	COXH_HUMAN	8.7	20/92	(KAGIFQSVK(R) (K)AYADFYR(N)	45
Cytochrome C oxidase polypeptide II	COX2_HUMAN	25.2	7.5/150	(R)LLDVDNRVVLPIEAPIR(M)	36
Delta-sarcoglycan	SGCD_HUMAN	32.1	3.45/52	(K)DGEIKLDAAK(I)	25
Desmin	DESM_HUMAN	53.4	27.29/829	(R)AQYETIAAK(N) (K)VSDLTQAANK(N) (R)KLLEGEESR(I) (R)VAELYEEELR(E) (R)FASEASGYQDNIAR(L) (R)INLPIQTYSALNFR(E) (R)VDVERDNLLDDLQR(L) (R)TFGGAPGFPLGSPLSSPVPFR(A) (R)TPSSYGAGELLDFSLADAVNQEFLTTR(T)	17
DnaK-type molecular chaperone HSPA5 precursor	gi 87528[c)]	78.0	3.7/49	(K)ITITNDQNR(L) (K)IINEPTAAAIAYGLDK(R)	12
Fatty acid binding protein	FABH_HUMAN	147	28.8/220	(K)SIVTLDGGK(L) (K)SLGVGFATR(Q) (R)ELIDGKLILTLTHGTAVCTR(T)	40
Gamma-sarcoglycan	SGCG_HUMAN	32.2	4.8/54	(R)LEGESEFLFPLYAK(E)	26
Glutathione S-transferase omega 1	GTO1_HUMAN	27.6	4.1/205	(K)VPSLVGSFIR(S)	33
Glyceraldehyde 3-phosphate dehydrogenase	G3P2_HUMAN	35.9	4.2/80	(R)VPTANVSVVDLTCR(L)	25
Histone H4	H4_HUMAN	11.4	13.8/98	(K)VFLENVIRDAVTYTEHAK(R)	44
Heat shock protein 20 kDa (HSP20)	HSB6_HUMAN	17.1	15.7/113	(R)APSVALPVAQVPTDPGHFSVLLDK(H)	37
Heat shock protein 27 kDa (HSP27)	HS27_HUMAN	22.8	20.5/212	(R)LFDQAFGLPR(L) (R)AQLGGPEAAKSDTAAK (R)AQLGGPEAAKSDETAAK(L)	30
Heat shock protein 60 kDa (HSP60)	CH60_HUMAN	61.1	6.8/105	(R)VTDALNATRT(A) (K)VGGTSDVEVNEK(K) (K)ISSIQSIVPALEIANAHR(K)	16
Heat shock protein 70 kDa (HSP70)	HS71_HUMAN	61.1	4.4/150	(K)DAGVIAGLNVLR(I) (R)IINEPTAAAIAYGLDKK(S)	16
Interferon-induced protein with tetratricopeptide	IFT4_HUMAN	55.9	1.4/54	(K)AIELFQR(V)	41
Isocitrate dehydrogenase	IDHP_HUMAN	50.9	10.8/1017	(K)ATDVADR(A) (K)CATITPDEAR(V) (R)NILGGTVFREPIICK(N) (K)LNEHFLNTTDFLDTIK(S)	20
Long-chain-fatty-acid CoA ligase	LCFA_HUMAN	78.3	1.3/27	(K)IGFFQGDIR(L)	13
Malate dehydrogenase	MDHM_HUMAN	35.5	5.6/80	(K)VAVLGASGGIGQPLSLLLK(N)	26
Metaxin 2	MTX2_HUMAN	29.7	8.4/76	(K)VPFIHVGNQVVSELGPIVQFVK(A)	31
Microsomal glutathione S-transferase 3	GST3_HUMAN	16.5	8.1/72	(R)IASGLGLAWIVGR(V)	39
Muscle-specific protein	Q9HB92[b)]	29.9	8.7/112	(K)YYQSPWEQAISNDPELLEALYPK(L)	26

Table 1. Continued

Name	Accession code[a)]	Mol. mass (kDa)	Sequence coverage %/ MOWSE score	Sequence	Band no.
Myosin light chain 1	MLE1_HUMAN	21.0	4.5/83	(K)HVLATLGE(K)	33
Myosin heavy chain	MYH7_HUMAN	223.7	8.84/1191	(R)QLLQANPILEAFGNAK(T) (K)LQVELDNVTGLLSQSDSK(S) (R)IAQLEEELEEEQGNTELINDRLK(K)	1
Myosin regulatory light chain 2	MLRV_HUMAN	18.6	16.2/178	(K)GADPEETILNAFK(L) (K)LK GADPEETILN AFK(V)	37
NADH-ubiquinone oxidoreductase 24 kDa subunit	NUHM_HUMAN	27.3	5.2/57	(K)AAAVLPVLDLAQR(Q)	32
Peripherin	PERI_HUMAN	53.9	2/111	(R)KLLEGEESR(I)	17
Peroxiredoxin 6	PDX6_HUMAN	24.9	7.6/371	(R)FHDFLGDSWGILFSHPR(D)	31
Phosphate carrier protein	MPCP_HUMAN	40.1	2.8/77	(K)GSSASLVLKR(L)	27
Prohibitin	PHB_HUMAN	29.8	24.26/690	(R) QVSDDLTER(A) (R)FDAGELITQR(E) (K)DLQNVNITLR(I) (R)KLEAAEDIAYQLSR(S) (R)IFTSIGEDYDERVLPSITTEILK(S)	29
Proteasome activator complex subunit 1	PSE1_HUMAN	28.7	10.8/425	(K)TENLLGSYFPK(K) (R)NAYAVLYDIILK(N) (R)NAYAVLYDIILKNFEK(L)	30
Protein-glutamine gamma-glutamyltransferase	gi 20141877[c)]	77.3	2/58	(R)YLLNLNLEPFSE(K)	11
Pyruvate dehydrogenase E_1 component beta	ODPB_HUMAN	39.2	3.1/86	(K)VVSPWNSEDAK(G)	25
Ras related protein rab-7	RAB7_HUMAN	23.5	6.8/23	(K)EAINVEQAFQTIAR(N)	34
Ras related protein rab-2	Gi 106185[c)]	23.6	6.5	(K)LQIWDTAGQESFR(S)	35
Regulator of G protein signaling protein RGS	Q8QHK0[b)]	25.6	5.91/41	(R)MIYEDYISILSP(K)	25
T complex protein 1 beta subunit	TCPB_HUMN	57.4	7.3/443	(K)LGGSLADSYLDEGFLLDKK(I)	16
Tetratricopeptide repeat protein II	TTCB_HUMAN	16.9	7.2/52	(K)GIVLLEELLPK(G)	41
Thioredoxin-dependent peroxide reductase	PDX3_HUMAN	27.7	5.5/45	(R)DYGVLLEGSGLALR(G)	32
Trifunctional enzyme alpha subunit	ECHA_HUMAN	82.9	2.7/100	(KTVLGTPEVLLGALPGAGGTQR(L)	
Troponin I	TRIC_HUMAN	23.9	21.1/850	(K)NITEIADLTQK(I) (R)CQPLELAGLGFAELQDLCR(Q)	30
Troponin T	TRT2_HUMAN	35.8	5.39/354	(K)DLNELQALIEAHFENR(K) (K)ELWQSIYNLEAEKFDLQEK(F)	26
Tubulin beta-9 chain	TBB9_HUMAN	49.6	5.6/83	(R)SGP FGQIFRPDNF VFGQSGAGNN WAK(G)	17
Ubiquinol-cytochrome C reductase	UCR6_HUMAN	13.4	15.5/517	(K)YEEENFYLEPYLKEVIR(E)	43
Vimentin	VIME_HUMAN	53.5	4.5/190	(R)FLEQQNK(I) (R)ISLPLPNFSSLNLR(E)	18
Voltage-dependent anion-selective channel	POR1_HUMAN	30.6	10.9/196	(R)VTQSNFAVGYK(T) (K)VNNSSLIGLGYTQTLKPGIK(L)	27
Voltage-dependent anion-selective channel	POR2_HUMAN	30.6	5.2/166	(K)LTLSALVDGK(S)	28
Voltage-dependent anion-selective channel	POR3_HUMAN	30.6	8.1/156	(K)LTLSALIDGK(N) (K)LTLDTIFVPNTGK(K)	28

a) Accession code in Swiss-Prot except where noted by footnotes (b, c).
b) Accession code in Trembl.
c) Accession code in NCBI.

3.3 Protein changes in human membrane microdomains during heart failure: 2-DE

Since this is the first investigation of membrane microdomains from human heart, pilot studies were performed to optimize and verify experimental parameters and indexes. Numerous modifications of the classic proteomics protocols exist to improve solubilization of protein mixtures, especially when dealing with hydrophobic (membrane) proteins [24, 25]. Therefore, pelleted membranes were first solubilized in a small volume of highly concentrated SDS resulting in efficient sample solubilization, and only thereafter were they diluted in IEF sample buffer to bring SDS content below the critical concentration that would interfere with IEF. In addition, this sample buffer contained thiourea, frequently used for membrane protein mixtures. Equal protein amount of membrane microdomains derived from individual failing subjects were subjected to 2-DE analysis, and compared with those derived from non-failing left ventricular tissues. Three 2-DE gels were performed for each sample, and their expression profiles were compared. In addition, to allow direct comparison of 2-DE gels, gels containing membrane microdomains from failing and non-failing subjects were paired, processed, and stained simultaneously.

An average of 30 protein spots with different expression levels were detected per gel. A total of 12 spots displayed decreased expression levels in the membrane microdomains derived from failing hearts compared with those derived from non-failing subjects, whereas 18 proteins displayed increased expression. Figure 4 shows the pattern of proteins of human heart membrane microdomains separated by 2-DE using a non-linear IPG IEF gradient pH 3–10. We analyzed the 30 differentially expressed spots by MS and identified all the proteins. The results are summarized in Table 2.

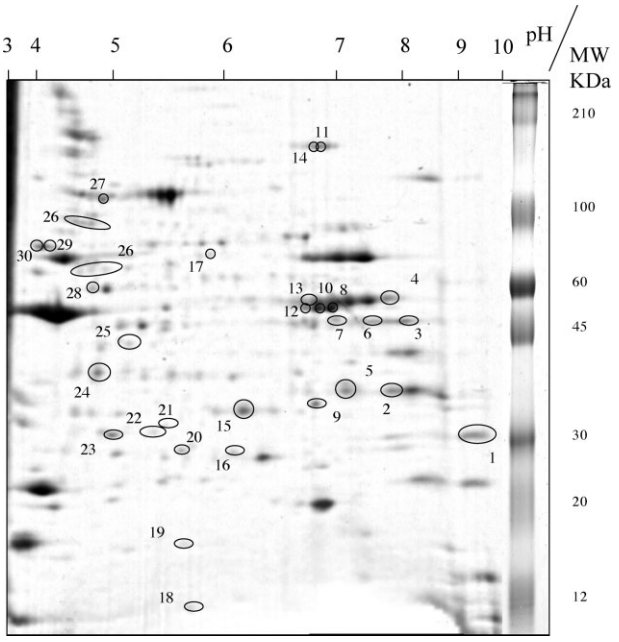

Figure 4. Representative 2-DE gel image of human heart membrane microdomains. Proteins (400 μg) were separated on IPG strips pH 3–10 and 7–17% acrylamide gradient gels. Spots showing significant differential expression ($p < 0.05$) in membrane microdomains derived from failing and non-failing human hearts are indicated by circles.

4 Discussion

This study represents the first example of subproteome analysis based on isolation and characterization of membrane microdomains of the failing and non-failing human heart. This approach not only allowed the study of otherwise undetectable proteins from cellular compartments, but also greatly improved the probability of detection and subsequent identification of proteins whose existence was merely postulated on the basis of their human genome sequence. The study has resulted in the identification by MS of the previously uncharacterized human heart membrane microdomain protein composition, thus allowing the comparative proteomic analysis of subset of proteins differently expressed in human heart failure. Our data indicated that human heart membrane microdomains are enriched in chaperones, cytoskeletal-associated proteins, enzymes and protein involved in the signal transduction pathway. In addition, the differential protein expression profile revealed the presence of proteins that were specifically up- or down-regulated in human heart failure. Among down-regulated proteins, αB-crystallin is a small heat shock protein (smHSP) structurally related to other smHSPs including αA-crystallin, HSP27, HSP20, HSP22, myotonic dystrophy protein kinase binding protein, and HSPB3 [26]. αB-crystallin has strong anti-apoptotic properties and, when fully induced, is the most abundant smHSP in the heart and may constitute as much as 5% of total cardiac myocyte protein. Moreover, ectopic overexpression of αB-crystallin protects cardiac myocytes from ischemic damage [27–29]. It has been proposed that chaperoning by αB-crystallin can stabilize myofilament proteins through selective interactions with actin, titin, nebulette and the intermediate filaments (IF), desmin and vimentin.

When αB-crystallin function is lost or compromised, abnormal desmin forms aberrant aggregates and disrupts the integrity of the desmin network, with pathological consequences [30, 31]. Altered distribution and organization of desmin filaments can indeed compromise cardiac function and induce heart remodeling [31, 32]. Increases in both desmin protein levels, as detected in our study, and in the desmin filament density in cardiac myocytes have been also observed in the progression of cardiac hypertrophy and failure from chronic pressure overload in guinea pigs [33]. An expression sequence tag study indicated that desmin was among the top six up-regulated genes in familial hypertrophic cardiomyopathic human hearts as well as in end-stage congestive heart failure [34, 35].

Table 2. Identification of differentially expressed proteins by MS

Name	Fold increased or decreased pathological vs control	Accession code[a)]	Mol. mass kDa/p*I*		Sequence coverage %/ MOWSE score	Sequence	Spot no.
			Theoretical	Experimental			
Decreased							
Acetyl CoA acetyltransferase	−6.8	THIL_HUMAN	45.2/8.98	45/7.1	3.9/246	(R)TPIGSFLGSLSLLPATK(L)	7
Acetyl CoA acetyltransferase	−11.9	THIL_HUMAN	45.2/8.98	50/6.9	13.1/455	(R)TPIGSFLGSLSLLPATK(L) (R)QAVLGAGLPISTPCTTINK(V) (K)AAWEAGKFGNEVIPVTVTVK(G)	8
Aconitate hydratase	−7.6	ACON_HUMAN	85.4/7.36	150/6.7	2.1/214	(K)IVYGHLDDPASQEIER(G)	14
Alpha crystallin B chain	−2.6	CRAB_HUMAN	20.2/6.72	33/6.7	35.4/1329	(R)FSVNLDVK(H) (K)HFSPEELK(V) (R)EEKPAVTAAPK(K) (R)TIPITREEKPAVTAAPK(K) (R)IPADVDPLTITSSLSSDGVLTVNGP(R) (K)YRIPADVDPLTITSSLSSDGVLTVNGPR(K)	9
Creatine kinase M chain	−11.9	KCRM_HUMAN	43.1/6.77	50/6.8	10.8/240	(R)GIWHNDNK(S) (K)LSVEALNSLTGEFKGK(Y) (R)LGSSEVEQVQLVVDGVK(L)	10
Creatine kinase	−4.6	KCRS_HUMAN	47.5/8.46	52/6.7	13.6/487	(R)EVENVAITALEGLK(G) (R)LGYILTCPSNLGTGLR(A) (R)EVENVAITALEGLKGDLAGR(Y) (R)SEVELVQIVIDGVNYLVDCEK(K)	13
Fatty acid binding protein	−3.2	FABH_HUMAN	14.7/6.34	13/5.75	28.8/220	(K)SIVTLDGGK(L) (K)SLGVGFATR(Q) (R)ELIDGKLILTLTHGTAVCTR(T)	18
Isocitrate dehydrogenase	−5.1	IDHP_HUMAN	50.9/8.88	51/8.0	10.8/937	(K)ATDVADR(A) (K)CATITPDEAR(V) (R)NILGGTVFREPIICK(N) (K)LNEHFLNTTDFLDTIK(S)	4
Troponin I	−2.7	TRIC_HUMAN	23.9/9.87	30/9.6	21.1/850	(R)AYATEPJAK(K) (K)NITEIADLTQKIFDLR(G) (R)CQPLELAGLGFAELQDLCR(Q)	1
Troponin T	−5.2	TRT2_HUMAN	35.8/4.94	47/7.5	11.8/254	(K)DLNELQALIEAHFENR(K) (K)ELWQSIYNLEAEKFDLQEK(F)	6
Voltage-dependent anion-selective channel	−4.2	POR1_HUMAN	30.6/8.63	35/8.0	34.7/883	(K)LTLSALLDGK(N) (K)YQIDPDACFSAK(V) (K)LTFDSSFSPNTGKK(N) (K)VNNSSLIGLGYTQTLKPGIK(L) (K)WNTDNTLGTEITVEDQLAR(G) (K)TDEFQLHTNVNDGTEFGGSIYQK(V)	2
Voltage-dependent anion-selective channel	−2.6	POR2_HUMAN	30.6/6.32	35/7.0	5.2/159	R)NNFAVGYR(T) (K)LTLSALVDGK(S)	5
Increased							
Creatine kinase	+9.1	KCRS_HUMAN	47.5/8.46	49/6.7	13.6/733	(R)LGYILTCPSNLGTGLR(A) (R)EVENVAITALEGLKGDLAGR(Y) (R)GTGGVDTAAVADVYDISNIDR(I)	12
Cytochrome C1	+2.1	CY1_HUMAN	35.4/9.15	32/6.8	17.8/911.13	(R)AANNGALPPDLSYIVR(A) (K)LFDYFPKPYPNSEAAR(A) (R)HGGEDYVFSLLTGYCEPPTGVSLR(E) (R)ARHGGEDYVFSLLTGYCEPPTGVSLR(E)	15

Table 2. Continued

Name	Fold increased or decreased pathological vs control	Accession code[a)]	Mol. mass kDa/p*I*		Sequence coverage %/ MOWSE score	Sequence	Spot no.
			Theoretical	Experimental			
Prohibitin	+5.4	PHB_HUMAN	29.8/5.57	30/5.1	24.26/690	(R) QVSDDLTER(A) (R)FDAGELITQR(E) (K)DLQNVNITLR(I) (R)KLEAAEDIAYQLSR(S) (R)IFTSIGEDYDERVLPSITTEILK(S)	23
Pyruvate dehydrogenase E_1 component beta	+5.3	ODPB_HUMAN	39.2/6.2	40/4.9	3.1/86	(K)VVSPWNSEDAK(G)	24
Troponin T	+16.2	TRT2_HUMAN	35.8/4.94	47/8.1	5.39/354	(K)DLNELQALIEAHFEN(K)	3
Aconitate hydratase	N.D.[b)]	ACON_HUMAN	85.4/7.36	150/6.8	3.2/469	(K)NTIVTSYNR(N) (K)QGLLPLTFADPADYNK(I)	11
Actin, alpha cardiac	N.D.[b)]	ACTC_HUMAN	42.1/5.23	44/5.2	26.7/1149	(K)AGFAGDDAPR(A) (R)AVFPSIVGRPR(H) (K)DSYVGDEAQSKR(G) (R)LDLAGRDLTDYLMK(I) (K)SYELPDGQVITIGNER(F)	25
Desmin	N.D.[b)]	DESM_HUMAN	53.4/5.21	70/4.0	26.44/2295	(K)VHEEEIR(E) (R)AQYETIAAK(N) (R)RIESLNEEIAFLK(K) (R)VDVERDNLLDDLQR(L) (K)VSDLTQAANKNNDALR(Q) (R)FLEQQNAALAAEVNRLK(G) (R)TFGGAPGFPLGSPLSSPVFPR(A) (R)TPSSYGAGELLDFSLADAVNQEFLTTR(T)	30
Glutathione S-transferase omega 1	N.D.[b)]	GTO1_HUMAN	27.6/6.24	31/5.6	4.1/160	(K)VPSLVGSFIR(S)	21
Heat shock protein 20 kDa (HSP20)	N.D.[b)]	HSBX_HUMAN	17.1/5.95	17/5.7	15.7/113	(R)APSVALPVAQVPTDPGHFSVLLDK(H)	19
Heat shock protein 27 kDa (HSP27)	N.D.[b)]	HSB1_HUMAN	22.8/5.98	28/5.7	24.4/189	(R)AQLGGPEAAKSDTAAK (R)VSLDVNHFAAPDELTVK(T) (K)LATQSNEITIPVTFESR(A)	20
Heat shock protein 70 kDa (HSP70)	N.D.[b)]	HS71_HUMAN	61.1/5.48	100/4.9	4.4/111	(K)DAGVIAGLNVLR(I) (R)IINEPTAAAIAYGLDKK(S)	27
Myosin heavy chain	N.D.[b)]	MYH6_HUMAN	223.7/5.60	60–80/4.5–5.2	1.9/347	(K)ITITNDKGR(L) (K)IINEPTAAAIAYGLDKK(G)	26
Peroxiredoxin 6	N.D.[b)]	PDX6_HUMAN	24.9/6.02	28/6.1	7.6/371	(R)FHDFLGDSWGILFSHPR(D)	16
Proteasome activator complex subunit 1	N.D.[b)]	PSE1_HUMAN	28.7/5.78	31/5.6	10.8/339	(K)TENLLGSYFPK(K) (R)NAYAVLYDIILK(N) (R)NAYAVLYDIILKNFEK(L)	22
T complex protein 1 beta subunit	N.D.[b)]	TCPB_HUMAN	57.4/6.02	65/5.9	7.3/443	(K)LGGSLADSYLDEGFLLDKK(I) (R)LALVTGGEIASTFDHPELVK(L)	17
Ubiquinol-cytochrome C reductase	N.D.[b)]	UCR1_HUMAN	52.6/5.94	58/4.8	15.6/816	(K)AVELLGDIVQNCSLEDSQIEKER(D) (K)YIYDQCPAVAGYGPIEQLPDYNR(I) (R)DVVFNYLHATAFQGTPLAQAVEGPSENV(R)	28
Vimentin	N.D.[b)]	VIME_HUMAN	53.5/5.06	70/4.2	4.5/190	(R)FLEQQNK(I) (R)ISLPLPNFSSLNLR(E)	29

a) Accession code in Swiss-Prot
b) Not determined indicates protein present only in pathological samples.

In this study, other HSPs were shown to be up-regulated in the membrane microdomain of heart failure myocardium, *i.e.*, HSP27, HSP70, and the not-previously described, HSP20. There is evidence that HSPs are also expressed at the cell membrane where they have been suggested to exert a necessary, but still poorly understood, role in the immune response and signal transduction [36, 37]. Different HSPs were found to be associated with membrane microdomains to a variable extent, depending on the particular HSP considered. Association of HSPs with the microdomains could be modulated by stress [36]. However, the exact role of HSPs in cardioprotective mechanisms against stresses remains unclear. The most interesting feature of their cytoprotective roles is that they are believed to act to stabilize cytoskeletal structure such as actin stress fibers and IF [38–40].

The presence of the cytoskeletal proteins in human heart membrane microdomains supports evidence from other cells that these domains regulate cytoskeletal assembly and are intrinsically linked to active movements of the cortical actin, subsequently regulating signal transduction [41–47]. Two sarcomeric actins, α-cardiac and α-skeletal actin, are coexpressed in normal adult myocardium and represent the predominant thin filament actin isoforms of cardiomyocyte contractile units [48]. To date, however, the knowledge of actin isoform expression in the heart during cardiac hypertrophy and failure is scarce and controversial [49–51]. Our data indicated that α-cardiac actin is the one protein that predominantly undergoes up-regulation in membrane microdomains of failing hearts.

In conjunction with two pairs of nonidentical light chains, two myosin heavy chains (MyHCs) constitute the functional myosin motor molecule. Two isoforms of MyHC (α and β) are expressed in mammalian heart. αMyHC has a higher ATPase activity than βMyHC [52, 53]. Our data indicate that there is a substantial increase in the amount of βMyHC in membrane microdomains of failing heart. That up-regulation of βMyHC expression can contribute to the development of myocardial dysfunction is supported by other studies, in which this maladaptation in the proportion of cardiac αMyHC and βMyHC isoforms can be reversed in animal models of heart failure after drug or surgical therapy [53–55].

Myofibrillar dysfunction in heart failure may also be caused by alterations in the properties and concentrations of troponins (Tns). Three subtypes of Tn exist: TnI (inhibitory), TnT (tropomyosin binding) and TnC (Ca^{2+} binding), all playing a specific role in the regulation of muscle contraction. Fine tuning of the functions of troponins *in vivo* occurs by phosphorylation of specific amino acids in specific domains of the molecules [56]. This subproteomic study first allowed the detection of Tn and the identification of PTM of TnT, with increased phosphorylation. Moreover, a reduction of TnI content occurs in membrane microdomains from failing heart. An increasing body of evidence points to PTMs of the thin filament regulatory proteins, cardiac TnT and TnI, as important in both long- and short-term regulation of cardiac function, and potentially implicated in the transition between compensated hypertrophy and decompensation [57].

Many mitochondrial proteins have been found in membrane microdomains, raising the intriguing possibility of the existence of mitochondrial rafts [58, 59]. However, we can not definitely exclude that partial contamination by mitochondrial proteins, which are highly enriched in heart, could be present in our membrane microdomain preparation. Our data show that human heart membrane microdomains contain the ATP synthase complex as well as mitochondrial respiratory proteins including NADH dehydrogenase, succinate dehydrogenase, cytochrome C oxidase, ubiquinol-cytochrome C reductase complex, and aconitate hydratase, implying that these microdomains could be a mitochondrial platform to integrate chemiosmosis as well as oxidation-reduction reactions. Some mitochondrial proteins such as voltage-dependent channel (VDAC), prohibitin, and ATP synthase have been described in association with either the plasma membrane or surface receptors [60, 61]. Interestingly, prohibitin, is observed, for the first time, to be strongly up-regulated in membrane microdomains derived from human failing heart. Prohibitin, an evolutionarily conserved protein with homologues from yeast to man, is involved in many cellular processes, such as cell cycle regulation, senescence, transcription regulation, tumor suppression, apoptosis and assembly of mitochondrial respiration chain complexes [62, 63]. However the role of prohibitin in heart failure remains unclear.

Interestingly our data show a reduction in VDAC levels in membrane microdomains of human heart failure. The presence of VDAC in the plasma membrane microdomains of different cell types [61] opens stimulating questions about their physiological role in these compartments. It might serve as the conduit for solute release from potocytotic vesicles. Mitochondrial VDAC has been shown to catalyze the translocation of DNA through artificial membranes [64], and it might perform a similar function in membrane microdomains. A second hypothesis on the plasma membrane targeting of VDAC relies on its affinity for cholesterol. VDAC might be present in membrane microdomains and could then be relocated to subcellular compartments through the intracellular cholesterol transport routes.

Interestingly, our data show a significant reduction in isocitrate dehydrogenase and aconitate hydratase in membrane microdomains derived from human failing heart. Although much remains to be learned about the role and regulation of mitochondrial isocitrate dehydrogenase in mammalian cells, and especially in the heart where it presents its highest activity and expression, recent data described a reduction in activity and expression during cardiac hypertrophy development in rats, supporting its relevance as an early and persistent marker of oxidative stress-related mitochondrial alterations in hypertrophy development [65, 66]. Moreover, the decrease in the levels of mitochondrial isocitrate dehydrogenase is consistent with the suggestion

that, in heart failure, the myocardium is unable to provide enough energy to cope with the increased mechanical stresses [67].

The antioxidant protein peroxiredoxin 6 (PRDX6), or anti-oxidant protein 2, is a member of a family of thiol-specific antioxidants, recently renamed peroxiredoxins. These proteins evolved as a part of a system that counteracts the damaging effects of oxygen radicals. PRDX6 is a unique nonredundant antioxidant that functions independently of other peroxiredoxins and antioxidant proteins [68]. It is intriguing that this study showed a significant up-regulation of PRDX6 in human heart failure, probably representing a defensive compensatory reaction to the oxidative damage.

Another protein of interest among those up-regulated in membrane microdomains of failing hearts is GST omega 1 (GSTO1). Laliberte *et al*. [69] report that current experimental drugs known as cytokine release inhibitory drugs, which inhibit interleukin-1β (IL-1β) post-translational processing, have GSTO1 as their direct target. This implies that GSTO1 may be involved in the activation of IL-1, and thus variation in GSTO1 could alter the efficacy of IL-1β post-translational processing, modulating an inflammatory response [69] in cardiovascular disease [70].

Among the metabolic changes observed in heart failure, a decreased in fatty acid oxidation is also evident [71]. A reduced fatty acid binding protein (FABP) content, as observed for the first time in this human heart subproteomic study, may account for the lowered fatty acid oxidation rate. Moreover, the important role of heart FABP in regulation of beta-oxidation in cardiac muscle was discovered with the creation of mice deficient in heart FABP, which had a severe defect in cardiac myocyte long-chain fatty acid cellular transport and beta-oxidation [72]. An interaction of FABP with anionic phospholipids and/or CD36 could promote the vectorial transport of fatty acids across the plasma membrane. It is also quite conceivable that FABP can provide fatty acids to preformed caveolar vesicles by protein-phospholipid or protein-protein interactions with caveolins [73].

In conclusion, this study, extending previous observations derived from global mapping of human protein expression performed on dilated cardiomyopathy [74], shows that subproteomic analysis of human heart membrane microdomains allowed the identification of multiple proteins, including both known and previously uncharacterized gene products, opening new perspectives for researcher to determine which role or roles they may play in any biological system or process such as human heart failure.

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