Amino Acids (2011) 41:401–414
DOI 10.1007/s00726-010-0675-3

ORIGINAL ARTICLE

Adaptation of proteomic techniques for the identification and characterization of protein species from murine heart

Karima Schwab · Boris Neumann · Christian Scheler · Peter R. Jungblut · Franz Theuring

Received: 5 March 2010 / Accepted: 24 June 2010 / Published online: 6 July 2010

Abstract Disturbed energy metabolism with impaired fatty acid oxidation, ATP synthesis and changing levels of contractile proteins has been observed during the development and manifestation of cardiovascular diseases, with the latter showing sexual differences in terms of onset, manifestation and progress. Estrogenic compounds, such as estrogens and phytoestrogens, are known to exert beneficial effects on several cardiovascular parameters. However, global studies implying the normal, non-failing myocardium are rare. Thus, identifying and characterizing protein patterns involved in the maintenance of normal heart physiology at the protein species level will help understanding disease conditions. In this study, we performed an adapted 2-DE/MS approach in order to identify and characterize post-translational modified and truncated protein species from murine heart. Female and male animals of different age were receiving the phytoestrogen genistein and comparative analyses were performed to identify sex and genistein treatment-related effects. Selected 2-DE spots that exposed varying abundance between animal groups and identified as identical proteins were subject to multi-protease cleavage to generate an elevated sequence coverage enabling characterization of post-translational modifications and truncation loci via high-resolution MS. Several truncated, phosphorylated and acetylated species were identified for mitochondrial ATP synthase, malate dehydrogenase and trifunctional enzyme subunit alpha. However, confirmation of several of these modifications by manual spectra interpretation failed. Thus, our results warrant caution for the blind trust in software output. For the regulatory light chain of myosin, we identified an N-terminal processed species, which so far has been related to ischemic conditions only. We tried to unravel the information content of protein species separated by high-resolution 2-DE as an alternative to high-throughput proteomics, which mainly is interested in lists of protein names, ignoring the protein species identity.

Keywords Cardiovascular diseases · Proteomics · Protein species · Post-translational modification

Introduction

Cardiovascular diseases (CVDs) are the number one cause of morbidity and mortality with an age and sexual divergence (Baker et al. 2003). Premenopausal women are at a lower risk for CVD as compared to age-matched men, but this risk increases dramatically after menopause, indicating that estrogens may play a protective role. However, hormone replacement therapy in humans yielded conflicting results (Hodis 2008) and phytoestrogens such as genistein, widely used in traditional asian medicine, could represent alternative compounds as they are known to exert estrogenic activity and to have beneficial effect on a wide range of cardiovascular parameters (de Kleijn et al. 2002; Kondo

Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0675-3) contains supplementary material, which is available to authorized users.

K. Schwab (✉) · B. Neumann · F. Theuring
Center for Cardiovascular Research, Institute of Pharmacology, Charité Universitätsmedizin, Berlin, Germany
e-mail: karima.schwab@charite.de

B. Neumann · C. Scheler
Proteome Factory AG, Berlin, Germany

P. R. Jungblut
Core Facility Protein Analysis, Max Planck Institute for Infection Biology, Berlin, Germany

et al. 2002; McCarty 2006; Squadrito et al. 2003). In the cardiovascular context, a disturbed energy metabolism with impaired fatty acid oxidation, ATP synthesis and changing levels of contractile proteins have been observed during diseased conditions (Ashrafian et al. 2007; Kuznetsov et al. 2009; Meng et al. 2009; Rottbauer et al. 2006; Stanley et al. 2005). Whereas numerous studies focussed on gene expression analyses at the messenger RNA level, other holistic and undirected techniques, such as proteomics, have been applied to the analysis of CVD (Knecht et al. 1994; Arrell et al. 2001; Jäger et al. 2002). Multiple identifications of a single protein from various spots on 2-DE gels revealed that the suggestion of a single gene or transcript, encoding for a single protein, is obsolete. The diversity in different forms of a protein emerging from one single protein-coding gene promoted the new term of *protein species* (Jungblut et al. 1996, 2008). While genomic and transcriptomic data lack information on protein species in a given tissue, advances in proteome analysis and mass spectrometry enabled the identification and characterization of post-translational modifications (PTMs) in peptides derived from a protein species, which lead to an increasing number of protein species in databases. PhosphositePlus, a database for phosphorylations and other PTMs in human and rodent proteins, lists about 700 known modifications of cardiac proteins, e.g. phosphorylation, acetylation and ubiquitinylation (http://phosphosite.org, February, 2010). Furthermore, advances in mass spectrometry allowed the localization of cleavage sites for protein processing, maturation, truncation and degradation. Protein species resulting from such cleavage events play an important role in inflammation, cell degeneration, apoptosis and oncogenesis (Dello and Rovida 2002). In the cardiovascular context, protein species derived from modifications such as acetylation, phosphorylation and cleavage are involved in various processes and disease development (Kao et al. 2006; Spiekerkoetter et al. 2003, 2004).

In this study, we analyzed the effects of a dietary supplement with the phytoestrogen genistein on the cardiac proteome pattern of young, adult and castrated male and female mice. Our analysis demonstrates considerable changes of the heart proteome with dietary genistein administration for both male and female animals. A changing abundance, of among others metabolic, energetic and contractile proteins, was observed (detailed data are presented elsewhere). However, here, we focussed on the identification of PTMs of four selected proteins, e.g. mitochondrial ATP synthase subunit alpha, trifunctional enzyme subunit alpha, malate dehydrogenase and cardiac myosin regulatory light chain 2. PTMs were identified by standard NanoLC electrospray ionization ion trap mass spectrometry (nanoLC-ESI–MS/MS) and linear ion trap Fourier transform ion cyclotron resonance mass spectrometry (LTQ-FT-ICR-MS/MS) and revealed several modified and truncated species.

Experimental procedures

Animals and diets

Male and female C57BL/6J mice were maintained on a phytoestrogen-free basic chow (HT 2014, Harlan Teklad, Germany) or basic chow enriched with 100 mg genistein/kg (genistein was supplied by Carl Roth, Germany and the enrichment was done by Harlan Teklad, Germany), resulting in an approximate oral dose of 10 mg genistein/kg body weight/day. To exclude unintended phytoestrogen influence, parent animals were maintained on a phytoestrogen-free chow 3 weeks before mating and throughout breeding. Animals had free access to food and water and were killed by cervical dislocation at different time points (intact 1, 3 and 6 months; gonadectomized 6 months) resulting in 16 study groups ($n = 7$ per group). All animal experiments were performed in accordance with the German Guidelines for the care and use of laboratory animals.

Gonadectomy

To study the effect of oral phytoestrogen treatment on the hormonal deficiency, animals were gonadectomized at the age of 3 months. Castration for female and male animals followed standard procedures (Lekgabe et al. 2006; Song et al. 2006).

Sample preparation

Hearts were excised, washed thoroughly several times in ice cold buffer (30 mM 2-*N*-morpholino ethane sulfonic acid, 60 mM sodium fluoride, 0.9% NaCl) to completely remove blood components and immediately frozen in liquid nitrogen. Samples for protein investigations were pestled in liquid nitrogen and stored at −80°C until further use.

Protein extraction

Total protein extract was obtained from crushed frozen tissue according to Klose and Kobalz (1995). 100 µg sample was incubated for 45 min in extraction buffer (7 M urea, 2 M thiourea, 2% ampholyte 2-4, 70 mM DTT, 25 mM Tris/HCl, 50 mM KCl, 3 mM EDTA, 2.9 mM benzamidine and 2.1 µM leupeptin) and centrifuged for 45 min at 12,000*g* and RT. The supernatant was transferred

to new tubes and the protein concentration was determined with the Bradford method.

Two-dimensional gel electrophoresis (2-DE)

Figure 1 illustrates the total 2-DE–MS workflow. Protein identification and data validation were carried out using ESI–MS/MS and LTQ-FT-ICR-MS/MS strategies. The samples were subjected to high-resolution 2-DE according to Klose and Kobalz (1995) with an updated protocol (Zimny-Arndt et al. 2009). Briefly 100 µg for analytical or 400 µg for preparative gels was anodically loaded, focussed at 8,500 Vh in a gradient between p*I* 2 and 11 and further separated with SDS–PAGE in 15% acrylamide gels. The final gel size is 20 cm × 30 cm. The 2-DE gels have a thickness of 0.75 or 1.5 mm for analytical and preparative gels, respectively. Gels were fixed in 50% ethanol and 10% acetic acid over night. Analytical gels were silver stained according to Heukeshoven and Dernick (1985), whereas preparative gels were stained in Coomassie Brilliant Blue R-250 (Eckerskorn et al. 1988).

Comparative gel analyses

Image analysis was performed with the Proteomweaver software version 3.0.9.9 (Biorad, Germany) according to the manufacturer's instructions. Manual spot editing and matching were carried out to reduce inaccurate spot matching. Relative spot intensities were statistically analyzed using a Student's *t* test. Comparative experiments were carried out for animals of the same age, same hormonal status, but different sex or different diet and resulted in 16 experiments as defined in Table 1. Differences were considered to be statistically significant at $p < 0.05$ with a regulation factor following the minimal significant factor calculated by the software (based on group size, standard deviation within one group, standard deviation between two groups and resulting trust factor). The minimal significant factors were for each experiment: 1MF, 1.50; 1MFG, 1.60; 1F, 1.50; 1M, 1.50; 3MF, 1.40; 3MFG, 1.55; 3F, 1.52; 3M, 1.38; 6MF, 1.33; 6MFG, 1.44; 6F, 1.37; 6M, 1.30; CASTMF, 1.25; CASTMFG, 1.44; CASTF, 1.50; CASTM, 1.50. Moreover, only spots present in at least five out of the seven gels in each study group were included.

Protein digestion

Spots of interest were excised from preparative gels and in-gel digested by 200 ng proteinase per spot over night. Reaction was stopped by acidification. Protein identification was carried out by ESI–MS analysis with tryptic peptides. To enhance sequence coverage, a parallel approach was performed with an LTQ-FT high-resolution MS instrument. Spots were taken in quadruplicates and each digested with one of the following proteinases: trypsin, chymotrypsin, AspN and thermolysine. After overnight digestion and acidification, digests were combined and applied to LTQ-FT-ICR-MS/MS.

NanoLC-ESI–MS/MS

The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Germany), a PicoTip emitter (New Objective, USA) and an Esquire plus ion trap MS (Bruker, Germany). After trapping and desalting the peptides on enrichment column (Zorbax SB C18, 0.3 mm × 5 mm, Agilent, Germany) using 1% acetonitrile and 0.5% formic acid solution for 5 min, peptides were separated on Zorbax 300 SB C18, 75 µm × 150 mm column (Agilent, Germany) using an acetonitrile and 0.1% formic acid gradient from 5 to 40% acetonitrile within 40 min. MS spectra were automatically taken by Esquire 3000 plus according to manufacturer's instrument settings for nanoLC-ESI–MS/MS analyses (ESI: scan range 100–3,000 *m/z*; MS^2 criteria: 2 most intensive ions in 300–1,600 *m/z*; target 35,000 ions; active exclusion of precursor ions after 2 spectra). Proteins were identified using MS/MS ion search of Mascot search engine Version No. 2.2 (http://www.matrixscience.com, Matrix Science, UK) and the Swiss Prot database (Swiss Prot 57.12, Swiss Institute of Bioinformatics, Switzerland). Parameters for database searching were: Mus musculus, type of search (MS/MS ion search), enzyme (trypsin), variable modifications (oxidation M, propionamide C, ester DE, acetylation K, phosphorylation ST, phosphorylation Y), mass values (monoisotopic), peptide mass tolerance (±0.1%), fragment mass tolerance (±0.5 Da), ion charge (1+, 2+ and 3+) and maximal missed cleavages (1). Proteins with a significant score of at least 40 and at least 2 significant peptide matches at the MSMS level have been considered as properly identified. For the identification of PTMs, an error tolerant search was performed. The UNIMOD database (http://www.unimod.org, Matrix Science, UK) was used to control mass differences introduced to the proteins.

LTQ-FT-ICR-MS/MS

An Agilent 1100 nanoLC system (Agilent, Germany) was coupled to an Advion NanoMate 100 chip-electrospray system (Advion, USA) and detection was performed on a Finnigan LTQ-FT mass spectrometer (ThermoFisher, Germany) equipped with a 6 T magnet. After trapping and desalting, the proteolytic peptides on enrichment column (Zorbax SB C18, 0.3 mm × 5 mm, Agilent) using 1% acetonitrile and 0.5% formic acid solution for

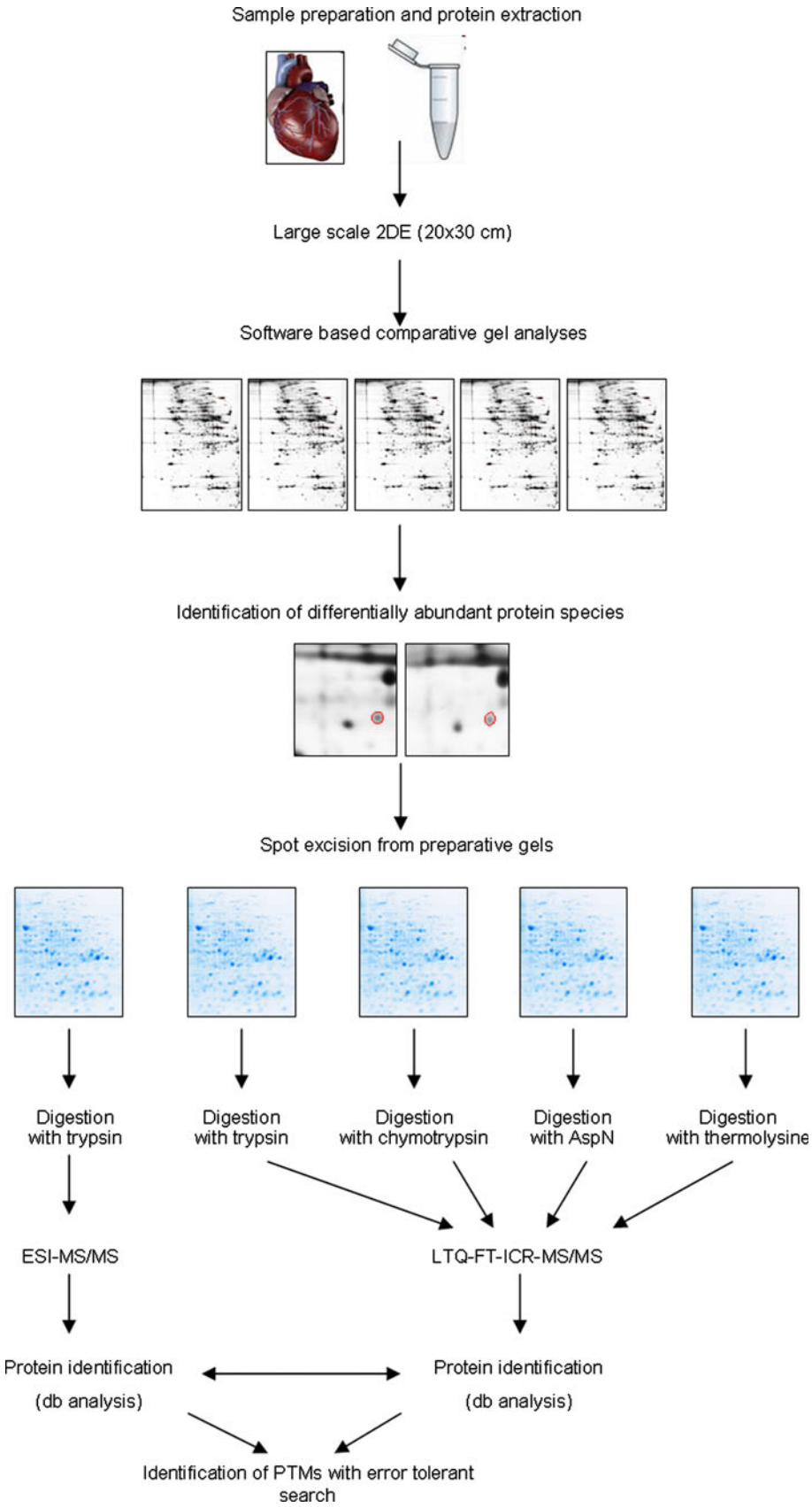

Fig. 1 Workflow of 2-DE (gel size 20 cm × 30 cm) and MS strategy for identification of proteins and protein species. *2-DE* two-dimensional gel electrophoresis, *ESI–MS/MS* electrospray ionization mass spectrometry, *LTQ-FT-ICR-MS/MS* linear ion trap Fourier transform ion cyclotron resonance mass spectrometry, *db* database

Table 1 Study design and compared study groups (experiments) in overview

Age	Experiment	Compared groups	Differentially abundant spots in total (a)	More abundant (b)	Less abundant (c)	Identified species (d)	Proteins (e)
1 month	1MF	M vs. F	9	4	5	9	9
	1MFG	M vs. F, genistein	16	4	12	15	12
	1F	F genistein vs. F	25	16	9	25	22
	1M	M genistein vs. M	21	6	15	16	16
3 months	3MF	M vs. F	33	19	14	25	23
	3MFG	M vs. F, genistein	82	66	16	69	54
	3F	F genistein vs. F	43	17	26	40	34
	3M	M genistein vs. M	62	48	14	49	41
6 months	6MF	M vs. F	62	27	35	56	43
	6MFG	M vs. F, genistein	48	18	30	43	36
	6F	F genistein vs. F	50	22	28	38	32
	6M	M genistein vs. M	70	37	33	63	47
6 months (CAST)	CASTMF	M vs. F	42	26	16	33	29
	CASTMFG	M vs. F, genistein	30	4	16	23	20
	CASTF	F genistein vs. F	23	16	7	15	13
	CASTM	M genistein vs. M	26	2	24	15	14

For sexually related differences, animals were compared within the same age, hormonal status but different sex. Animals of the same age, same sex and different diets were matched for diet-related differences. Comparative 2-DE results displaying the total number of differently abundant protein spots (a), thereof more abundant (b) and less abundant (c). The number of identified protein species (d) and the corresponding proteins (e) are also listed

M male, *F* female, *G* genistein, *CAST* castrated

5 min peptides were separated on Zorbax 300 SB C18, 75 µm × 150 mm column (Agilent, Germany) using an acetonitrile and 0.1% formic acid gradient from 5 to 40% acetonitrile within 40 min. MS overview spectra were automatically taken in FT mode according to manufacturer's instrument settings for high-resolution nanoLC-ESI–MS analyses [scan low mass: 350.00; scan high mass: 1,800.00; scan mode: FTMS + p NSI full ms (350.00–1,800.00); FT resolution: 100,000; CID of 5 most intensive peaks from (350–1,800) scan; automatic gain control was used with a target of 5e5 ions for FTMS full scans and 1e4 ions for MS^2 CID scans in the ion trap]. Peptide fragmentation and detection was accomplished in the instrument's LTQ ion trap. Proteins were identified using MS/MS ion search of Mascot search engine Version No. 2.2 (http://www.matrixscience.com, Matrix Science, UK) and the Swiss Prot database (Swiss Prot 57.12, Swiss Institute of Bioinformatics, Switzerland). Parameters for database searching were: *Mus musculus*, type of search (MS/MS ion search), enzyme (none), variable modifications (oxidation M, propionamide C, me-ester DE), mass values (monoisotopic), peptide mass tolerance (3 ppm), fragment mass tolerance (±0.3 Da), ion charge (1+, 2+ and 3+) and maximal missed cleavages (1). Protein and PTM identification criteria were identical to ESI–MS/MS.

Results

Differentially abundant protein spots

In order to characterize the cardiac protein patterns for male and female animals and to identify differentially regulated proteins after oral genistein administration, we performed large-scale 2-DE. Generated gels were highly reproducible, displaying more than 1,200 protein spots. For all comparative study groups, a total number of 434 protein spots were found to be differentially abundant. Out of those, 357 species were successfully identified with ESI–MS/MS (82%) and resulted in 209 different proteins, since one protein can be found in several different spots. An overview of the experimental design and the results is shown in Table 1 including differently abundant protein spots (more and less abundant), number of identified species and number of corresponding proteins. Classification of the proteins according to their biological process was conducted using the panther classification system database (http://www.pantherdb.org). Most changes were found for proteins involved in the maintenance of metabolic, transport and developmental processes, as well as for proteins linked to muscle contraction and energy generation. In this paper, we aimed to combine methods for accurate protein identification with high sequence coverage and to be able

to properly identify occurring PTMs in several protein species. Numerous proteins were present as several species. In the next section, we focus on MS data of four selected proteins: mitochondrial ATP synthase subunit alpha (ATPA), mitochondrial trifunctional enzyme subunit alpha (ECHA), mitochondrial malate dehydrogenase (MDHM) and ventricular/cardiac isoform, as well as atrial isoform of myosin regulatory light chain 2 (MLRV and MLRA).

Protein and PTM identification based on ESI–MS/MS

Based on the ESI ion trap tandem MS data (Tables 2, 3), we found ATPA, ECHA, MDHM and MLRV to be present in nine, four, seven and four different protein spots, respectively. The identified protein species are illustrated in Fig. 2. Some of the protein species showed changing abundance for several experiments and others were specific for certain comparative analyses (Table 2).

In the 6-month-old male mice, ATPA varied in six spots due to genistein treatment (experiment 6M, Table 2). Abundance of species 1, 2, 5 and 7 was enhanced and abundance of species 3 and 6 was lowered. However, female animals of the same age displayed spots 4 and 7 being elevated and spot 9 being decreased due to genistein treatment (experiment 6F, Table 2). Species 1, 2, 4, 5 and 7 are located at the expected molecular weight. Species 3, 6, 8 and 9 appeared at lower molecular weights (Fig. 2; Table 3). MS data revealed for these spots a truncation at the C-terminus (Table 3). The software suggested a pyro-phosphorylation for species 7 at T48; however, manual interpretation revealed phosphorylation at S52 or S53.

ECHA varied in four protein spots (Table 2). In castrated animals, male mice displayed a lower relative amount of this enzyme compared to female animals (experiment CASTMF, spot 11, Table 2). However, genistein treatment provoked an elevation of ECHA abundance as displayed in spots 10 and 13 (experiment CASTMFG, Table 2). All four identified ECHA species appeared at the expected molecular size, with species 12 being the most acidic one (Fig. 2; Table 3).

MDHM was identified in seven protein spots (Table 2). Species 16 and 18–20 displayed the expected size. For species 14 and 17, we detected a C-terminal cleavage, whereas species 15 was truncated at the N-terminus (Fig. 2; Table 3). Comparing male to female animals, 6 months old, an elevated abundance for MDHM was observed (experiment 6MF, spot 14, Table 2). Castration reduced MDHM abundance, which was independent from genistein supplementation (experiment CASTMF, spots 14 and 19; experiment CASTMFG, spot 18, Table 2). For MDHM species, no modification could be detected (Table 3).

The sarcomere protein MLRV was detected in four different species. Variable levels of MLRV were observed in several study groups. Male mice exerted decreased relative amounts of MLRV compared to female mice, independent from age, hormonal status or genistein treatment (experiments CASTMF, CASTMFG, 1MFG and 3MF, spots 22–24, Table 2). Out of the identified species, spot 22 was phosphorylated at S14 or S15 based on normal search. The error tolerant search revealed for the measured peptide a double phosphorylation as well as an N-terminal substitution with hexose (supplementary figure). However, it is much more logic that both N-terminal amino acids (i.e. were measured in the normal search) were not measured and the peptide is monophosphorylated (which results in the same mass).

Spot 24 displayed a 13 kDa fragment, resulting from N-terminal cleavage. In addition, this spot was phosphorylated, but phosphorylation locus could not be specified and occured at S14 or S15 (Fig. 2; Table 3).

Protein and PTM identification based on LTQ-FT-ICR-MS/MS

For data validation, protein species of interest were subjected to LTQ-FT-ICR-MS/MS. To increase sequence coverage, four different overnight digestion procedures were applied. Digests were combined and then applied to the high-resolution mass spectrometer (Fig. 1).

ATPA identification was confirmed for seven out of nine species. For species 5 and 9, ATPA was only matched as the second or third hit (supplementary Table 2). However, species 2, 4 and 7 showed acetylations at S106 (Table 4).

ECHA validation revealed a mismatch for spot 12. This spot was identified as 6-phosphofructokinase (K6PF). Both MS strategies were consistent for the other three protein species but displayed different PTMs, with LTQ-FT-ICR-MS/MS showing species 10 and 13 to be acetylated at K255 and K415, respectively (Table 4).

For MDHM, LTQ-FT-ICR-MS/MS-based identification (Table 4) resulted in one confirmed spot (20), three differently matched proteins (14, 15 and 17) and three other MDHM matches as a second hit (16, 18 and 19). Thus, spots 14, 15 and 17 were identified as ATPA, peroxiredoxin 1 (PRDX1) and cardiac troponin I (TNNI3), respectively. No modification was detectable for MDHM.

Regarding protein identification for MLRV, standard ESI–MS/MS- and LTQ-FT-ICR-MS/MS-based measurements were in agreement for three spots. Spot 23 was identified as MLRA by LTQ-FT-ICR-MS/MS. Moreover, phosphorylation at S15 for spot 22 was confirmed (Table 4).

Complementarity of ESI–MS and LTQ-FT-ICR-MS

Identification of protein spots of interest by standard ESI–MS resulted in 24 matched proteins, namely ATPA (spots

Table 2 Regulation of protein species from four proteins ATP synthase subunit alpha, mitochondrial (ATPA), trifunctional enzyme subunit alpha, mitochondrial (ECHA), malate dehydrogenase, mitochondrial (MDHM) and myosin regulatory light chain 2, ventricular isoform (MLRV) were found in nine, four, seven and four protein spots, respectively

Protein	Experiment (defined in Table 1)	Spot ID	Regulation factor	*p* value
ATPA_MOUSE (ID 1–9), number of identified species: 9, MW (Da): 59,716, p*I*: 9.22	1MFG	3	0.61	0.046
		5	1.64	0.001
		Spots 1, 2, 4, 6–9 not significantly different		
	3MFG	8	2.95	0.000
		Spots 1–7 and 9 not significantly different		
	3F	7	1.52	0.043
		Spots 1–6, 8 and 9 not significantly different		
	6MF	8	0.69	0.036
		Spots 1–7 and 9 not significantly different		
	6F	4	1.47	0.034
		7	1.38	0.044
		9	0.59	0.025
		Spots 1–3, 5, 6 and 8 not significantly different		
	6M	1	1.38	0.002
		2	1.34	0.037
		3	0.33	0.017
		5	1.40	0.004
		6	0.58	0.005
		7	1.55	0.012
		Spots 4, 8 and 9 not significantly different		
ECHA_MOUSE (ID 10–13), number of identified species: 4, MW (Da): 82,617, p*I*: 9.24	3MF	12	2.04	0.004
		Spots 10, 11 and 13 not significantly different		
	3MFG	11	1.70	0.016
		Spots 10, 12 and 13 not significantly different		
	3F	12	0.59	0.020
		Spots 10, 11 and 13 not significantly different		
	3M	13	1.91	0.002
		Spots 10, 12 not significantly different		
	6MFG	10	1.69	0.019
		Spots 11–13 not significantly different		
	6M	13	0.55	0.049
		Spots 10–12 not significantly different		
	CastMFG	10	1.72	0.028
		Spots 11–13 not significantly different		
	CastMFG	13	1.88	0.040
		Spots 10–12 not significantly different		
	CastMF	11	0.44	0.005
		Spots 10, 12 and 13 not significantly different		
MDHM_MOUSE (ID 14–20), number of identified species: 7, MW (Da): 35,589, p*I*: 8.93	3MF	16	1.77	0.020
		Spots 14–15 and 17–20 not significantly different		
	3MFG	20	0.64	0.013
		Spots 14–19 not significantly different		
	3F	17	0.49	0.030
		Spots 14–16 and 18–20 not significantly different		
	3M	15	1.63	0.038
		17	1.78	0.050
		Spots 14, 16 and 18–20 not significantly different		
	6MFG	14	1.57	0.013
		Spots 15–20 not significantly different		

Table 2 continued

Protein	Experiment (defined in Table 1)	Spot ID	Regulation factor	*p* value
	6F	14	1.75	0.039
		17	0.53	0.003
		Spots 15, 16 and 18–20 not significantly different		
	CastMF	14	0.48	0.010
		19	0.53	0.010
		Spots 15–18 and 20 not significantly different		
	CastMFG	18	0.62	0.036
		Spots 14–17, 19 and 20 not significantly different		
MLRV_MOUSE (ID 21-24), number of identified species: 4, MW (Da): 18,852, p*I*: 4.86	1MFG	24	0.47	0.030
		Spots 21–23 not significantly different		
	1F	22	0.60	0.015
		24	2.34	0.009
		Spots 21 and 23 not significantly different		
	3MF	24	0.41	0.000
		Spots 21–23 not significantly different		
	6F	21	1.38	0.024
		Spots 22–24 not significantly different		
	CastMF	22	0.66	0.020
		Spots 21, 23 and 24 not significantly different		
	CastMFG	23	0.50	0.007
		Spots 21, 22 and 24 not significantly different		
	CastF	23	2.19	0.004
		Spots 21, 22 and 24 not significantly different		

These spots are numbered (24 spots in total) and the experiments they were found to be changing are listed with the regulation factor and the corresponding *p* values. Identification is based on standard ESI–MS. Protein and entry names are corresponding to the Uni Prot database

1–9), ECHA (spots 10–13), MDHM (spots 14–20) and MLRV (spots 21–24). The sequence coverage ranged between 6 and 32%.

Data validation by LTQ-FT-ICR-MS with a multi-protease digestion procedure revealed for ATPA beside confirmed protein spots (spots 1–4 and 6–8), a colocalization of ATPA and MMSA and ATPA and PECI, for the spots 5 and 9, respectively. Moreover, peptide matches and total sequence coverage were enhanced (supplementary Tables 1 and 2).

Four spots were matched by standard ESI–MS to ECHA and three have been confirmed by LTQ-FT-ICR-MS with enhanced peptide matches and sequence coverage, due to multi-digestion (spots 10, 11 and 13). Spot 12 was identified as ECHA by standard ESI–MS with 14 matched peptides and 44% sequence coverage of the parent protein. However, LTQ-FT-ICR-MS identified this spot as K6PF (supplementary Tables 1 and 2).

LTQ-FT-ICR-MS confirmed MDHM identification for the spots 16 and 18–20 and identified additionally a colocalization with G3P in the spots 16, 18 and 19. This colocalization was not apparent by standard ESI–MS. However, spots 14, 15 and 17 were not identified as MDHM by LTQ-FT-ICR-MS, although peptide and sequence coverage by standard ESI–MS were ranging from 10 to 40 matched peptides and 33–61% sequence coverage of the parent protein (supplementary Tables 1 and 2).

The results of the spots 21–24, identified as MLRV by standard ESI–MS, were confirmed by LTQ-FT-ICR-MS approach for three spots and resulted in an increased number of the matched peptides and sequence coverage for the identified protein species (supplementary Tables 1 and 2). A possible colocalization of MLRA and MLRV was observed.

Regarding identified PTMs, standard ESI–MS and LTQ-FT-ICR-MS revealed almost always different modifications. Particularly for ATPA, standard ESI–MS measured a single modified species, whereas LTQ-FT-ICR-MS showed three species to be modified. For ECHA, spot 10, ESI–MS revealed no modifications but LTQ-FT-ICR-MS an acetylation at K255. Thus, standard ESI–MS and LTQ-FT-ICR-MS seem to deliver complementary results.

Manual data interpretation

As the Mascot-based PTM search in error tolerant mode is error prone, MS and MS*n* spectra of all spots were further applied to manual interpretation of spectra with

Table 3 Identification of post-translational modifications by ESI ion trap MS/MS and truncation loci for the proteins ATPA (ID 1–9), ECHA (ID 10–13), MDHM (ID 14–20) and MLRV (ID 21–24)

Protein	ID	Appearance on the 2-DE gel	Apparent size on the 2-DE gel (kDa)	Covered AA in parent protein	Modification	Modified peptide	Score	Mass (Da)	Mass error (%)	Mass error (ppm)
ATPA, 553 AA, 1–43 transit peptide	1	Intact	60	46–416						
	2	Intact	60	46–553						
	3	Degraded	30	46–230						
	4	Intact	60	46–316						
	5	Intact	60	46–472						
	6	Degraded	30	46–316						
	7	Intact	60	46–553	p-S52 and p-S53	TAEMpSpSILEER	70	1,424.5295	−0.05645	564.5
	8	Degraded	32	46–316						
	9	Degraded	40	58–503						
ECHA, 763 AA, 1–36 transit peptide	10	Intact	80	23–759						
	11	Intact	80	39–759						
	12	Intact	80	166–759						
	13	Intact	80	167–660						
MDHM, 338 AA, 1–24 transit peptide	14	Degraded	28	27–281						
	15	Degraded	20	166–324						
	16	Intact	35	27–324						
	17	Degraded	30	27–281						
	18	Intact	35	27–324						
	19	Intact	35	27–324						
	20	Intact	35	27–324						
MLRV, 166 AA	21	Intact	19	31–166						
	22	Intact	18	9–166	S15-p	GGSpSNVFSMFEQTQIQEFK	65	2,500.9747	0.05332	533.2
	23	Intact	18	59–104						
	24	Degraded	13	10–166	S14-p or S15-p	GGpSpSNVFSMFEQTQIQEFK	29	2,485.0873	0.01573	157.3

Only modifications confirmed by manual spectra interpretation are presented. Peptide sequence, peptide mass, mass error and peptide ion score of modified peptides are given

focus on suggested PTMs. Many of the suggested PTMs could not be confirmed and an example of misleading modifications for ECHA (spot 10) is presented: The software-based error tolerant search revealed for matched peptides beside acetylation at K166 (+42.01 Da) an additional amino acid substitution (−42.05 Da), resulting in no net mass change and thus the suggested acetylation could not be confirmed. For another peptide covering the amino acids 518–531 of the parent protein, the software suggested DAET at S518. In the UniMod database, DAET is classified as chemical derivative and described as the beta-elimination of a phosphoryl residue to amine thiol where the derivatization is done under strong alkaline conditions. This illustrates the need for manual data verification, especially when multiple modifications are affecting a single peptide. Using an error tolerant mode can help finding undocumented modifications but may mislead researchers by ambiguous results.

Spectra of all confirmed peptides carrying modifications are shown as supplementary figure.

Discussion

Our study revealed a complex influence of dietary genistein on the proteome of the murine heart. Depending on age and sex of the animals, the study provided insights into networks of interlinked protein species abundance profiles for the different animal groups. A changing abundance, of among others metabolic, energy generating and sarcomere proteins, such as ATPA, ATPB, MDHM, ECHA, ECHB, MLRV and MLRA, was observed. Detailed results on the

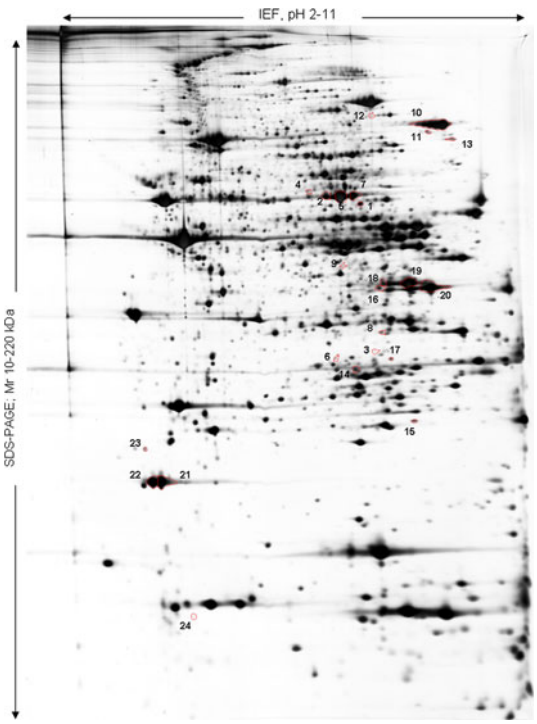

Fig. 2 2-DE protein pattern of the murine myocardium. Gels were loaded with 100 µg total cell extract and stained with silver. Protein species were identified based on ESI–MS/MS. *1–9* ATPA, *10–13* ECHA, *14–20* MDHM, *21–24* MLRV, *Mr* molecular range, *IEF* isoelectric focusing

impact of sex and genistein treatment are given elsewhere (manuscript submitted).

ATP synthase subunit alpha, mitochondrial (ATPA)

ATPA is a mitochondrial, membrane bound enzyme of the respiratory chain complex V, producing energy from ADP in the presence of a proton gradient. This gradient is generated by the complexes I–IV of the respiratory chain. Complex V consists of, among others, the subunits alpha and beta (ATPA and ATPB), which form the catalytic core. For ATPA, beside a transit peptide (amino acids 1–43), the residues 212–219 serve as a nucleotide binding site and residue 413 is required for activity (Yotov and St Arnaud 1993). A disturbed energy metabolism with impaired fatty acid oxidation and lower abundance of proteins involved in ATP synthesis has been observed during myocardial hypertrophy (Schott et al. 2008). It has been shown that ATPA can be modified to obtain different protein species. Phosphorylation at S76 in the brain of mice has been reported by Vosseler et al. (2005). In our experiment, we measured for species 7 two phosphorylations at S52 and S53, which can contribute to enzyme activity. Kim et al. (2006) reported ATPA acetylations in several lysine residues in mitochondria from mouse livers. We found species 2, 4 and 7 to be acetylated at S106. Enzymatic activity is regulated not only by additional modifications, but also through protein truncation. C-terminal cleaved species of ATPA, lacking the active site, were decreased in male and female mice due to genistein treatment. The reduced amount of cleaved enzyme might enhance total enzyme activity. Moreover, some intact species with several modifications were measured in animals of both sexes and could be related to enhanced activity. Since male and female mice displayed an elevated body weight due to genistein treatment (experiments 6F and 6M, significant level only in male mice, data not shown), we assume that oxidation of fatty acids and consequential elevation of ATPA activity might have occurred.

Trifunctional enzyme subunit alpha, mitochondrial (ECHA)

The mitochondrial trifunctional enzyme complex is composed of the subunits alpha and beta (ECHA and ECHB) and catalyzes three steps of the beta-oxidation, whereas ECHA is a bifunctional subunit. Thereby, long chain acyl-CoA is degraded to medium and short chain acyl-CoA (Spiekerkoetter et al. 2003, 2004). Variations at the protein and activity levels are associated with several cardiac dysfunctions, e.g. arrhythmia and cardiomyopathy (Kao et al. 2006). The residues E151 and E173 of ECHA are important for catalytic activity. We found ECHA in four different intact species, covering both catalytic sides. Decreased levels of full length ECHA and ECHB, as well as increased amounts of truncated ECHA species, have been associated with development of hypertension (Meng et al. 2009). Comparing male to female animals (independent from genistein intake), intact male mice exerted higher levels for ECHA than intact female mice. However, castration lowered this value but genistein supplementation prevented the reduction. Again the results were in accordance with the body weight of the animals, showing castrated males to lose weight and to recover upon genistein treatment (data not shown). Regarding modifications, acetylations at K255 for spot 10, as well as acetylation at K415 for spot 13 were measured. Many of the software suggested modifications for ECHA, even known in literature, were not taken into account, as the quality of the spectra was not satisfying. Thus, improvement of protein sequence coverage and identification of modifications are still needed.

Malate dehydrogenase, mitochondrial (MDHM)

MDHM is an enzyme of the Krebs' cycle and catalyzes the conversion of malate to oxaloacetate producing an equivalent amount of NADH. Residue H200 represents the active

Table 4 Identification of post-translational modifications by LTQ-FT-ICR-MS/MS and truncation loci for the proteins ATPA (ID 1–9), ECHA (ID 10–13), MDHM (ID 14–20) and MLRV (ID 21–24)

Protein	ID	Appearance on the 2-DE gel	Apparent size on the 2-DE gel (kDa)	Covered AA in parent protein	Modification	Modified peptide	Score	Mass (Da)	Mass error (ppm)
ATPA, 553 AA, 1–43 transit peptide	1	Intact	60	46–524					
	2	Intact	60	46–553	S106-a	MaSLNLEPDNVGVVVFGNDK	86	2,104.0147	5
	3	Degraded	30	46–374					
	4	Intact	60	46–553	S106-a	MaSLNLEPDNVGVVVFGNDK	81	2,104.0147	4
	5	Intact	60	63–551					
	6	Degraded	30	46–553					
	7	Intact	60	46–553	S106-a	MaSLNLEPDNVGVVVFGNDK	88	2,104.0147	5
	8	Degraded	32	46–350					
	9	Degraded	40	74–347					
ECHA, 763 AA, 1–36 transit peptide	10	Intact	80	44–759	K255-a	KGLADRaKVSAKQSK	18	1,556.8998	−3
	11	Intact	80	167–759					
	12	Intact	80						
	13	Intact	80	72–759	K415-a	KKaKALTSFERDSIFSNLIGQLDYKGF	9	3,126.5791	−4
MDHM, 338 AA, 1–24 transit peptide	14	Degraded	28						
	15	Degraded	20						
	16	Intact	35	27–324					
	17	Degraded	30						
	18	Intact	35	27–324					
	19	Intact	35	27–324					
	20	Intact	35	27–338					
MLRV, 166 AA	21	Intact	19	9–166					
	22	Intact	18	9–166	S15-p	IEGGSpSNVFSM	54	1,206.4628	−1
	23	Intact	18	14–166					
	24	Degraded	13	19–155					

Only modifications confirmed by manual spectra interpretation are presented. Peptide sequence, peptide mass, mass error and peptide ion score of modified peptides are given

site as a proton acceptor (Minarik et al. 2002). Decreased levels of MDHM have been associated with ischemic conditions in the heart to counteract excess proton accumulation (Kim et al. 2006). Multiple acetylation sites have been reported, whereas acetylations at K239 and K314 were observed in liver mitochondria of fasted animals (Kim et al. 2006). Suggested acetylation at lysine K239 in 2 species (14 and 17), with both species being truncated at the C-terminus, could not be confirmed by manual data interpretation. However, fasting conditions, acetylation at K239 and protein cleavage could be closely related and the acetylation of the species 14 and 17 needs clarification in more details. Differences between standard ESI–MS/MS- and LTQ-FT-ICR-MS/MS-based protein identification for MDHM might be related to protein and species colocalization. Improvement in the separation protocol, e.g. different pH gradient, might be useful to overcome these difficulties.

Myosin regulatory light chain 2, ventricular and atrial isoforms (MLRV and MLRA)

The cardiac myofilament consists of thin and thick filaments, which in striated muscle are highly regularly arranged. The thick filament is mostly composed of heavy and light myosin chains. The N-terminal part of the heavy chain contains ATPase activity, binds to actin thin filaments and builds a cross bridge for force generation (de Tombe 2003). Each myosin heavy chain binds one regulatory and one essential light chain, which influence the spatial arrangement of myosin heads in the thick filament. MLRV and MLRA can exist in phosphorylated or non-phosphorylated forms (MLRV: S15-p and MLRA: S22-p and S23-p). Phosphorylation at serine residues results in the addition of negative charge to the N-terminal region of MLRV and MLRA and causes the myosin heads to swing out from the thick filament

backbone toward the actin filament and thereby increases the probability of attachment to actin and promotes force generation (White et al. 2003). In diseased conditions, impaired myocardial function occurs through altered levels of contractile proteins (Foster and Van Eyk 1999), reduced levels of MLRV (Casey et al. 2005), altered MLRV and MLRA phosphorylation (Walker et al. 2009; Hammer et al. 2010) and the appearance of a truncated MLRV species missing the N-terminal part (White et al. 2003). Our study revealed three protein spots identified as MLRV and one species identified as MLRA, including an intact, phosphorylated MLRV species (species 22), as well as a phosphorylated and cleaved MLRV species (species 24) missing the N-terminal amino acids. Modification for spot 22 was measured based on both MS strategies, namely phosphorylation at S15. This phosphorylated species exhibited a lower p*I*, probably due to the additional phosphoric acid residue. Whether the decrease of the MLRV phosphorylated species in castrated male compared to female mice is related to disease needs to be elucidated in further studies.

The number of publications elucidating functional differences between protein states of interest (e.g. healthy and disease, treated and untreated) increased over the last years and resulted in improvement of liquid chromatography (LC), 2-DE and MS (Schluter et al. 2009). However, the challenge was to obtain 100% sequence coverage of peptides to be able to elucidate entire chemical modifications at the protein species level as presented for the protein ESAT6 of *Mycobacterium tuberculosis* (Okkels et al. 2004). The bottom-up approach, where proteins are first digested and then applied to LC and MS, allows the detection of PTMs, but discrimination between species is not possible (Hoehenwarter et al. 2006). On the other hand, the top-down approach allows identification of PTMs, but the strategy is limited to low mass proteins, since the protein mixture is directly subjected to LC (Schluter et al. 2009). The combination of both strategies, 2-DE–MS, separates protein species in spots. The spots are digested, further separated by nanoLC and the MS-based identification at the peptide level is accurate and sensitive. Moreover, using different digestion procedures can enhance the sequence coverage and thus allows the identification of occurring PTMs (Schluter et al. 2009). However, our applied multi-digest protocol in the 2-DE approach did indeed increase sequence coverage, but never achieved 100% (highest SC 95%). This could be related to unmeasured peptides not eluting from the LC column due to unspecific binding with the separation material. Moreover, colocalization of protein species (e.g. spots 1 and 10) and even proteins (spot 16, 18 and 19) were observed, thus improvement in the separation protocol is still needed. Nevertheless, coupling 2-DE separated and digested spots to sensitive MS is still so far the most appropriate approach to elucidate chemical modifications at the protein species level. However, one should always be aware to software suggested modifications and so far manual data validation and interpretation are still needed. Our work demonstrated once more the necessity to focus on functional characterization of protein species in comparative proteomics.

Conclusions

Protein species resulting from all protein modifications, post-translational chemical modifications and protein truncation are different products of one single gene. These modifications influence subcellular location, degradation, subunit assembly, tertiary structure or enzyme activity and thus protein function. Therefore, prime importance should be rather given to systematically identify and specify proteins at their species level than to quantify total protein amount. This study elucidated acetylation and phosphorylation sites, as well as a C-terminal cleaved species for ATPA. ECHA carried several acetylations, but no modifications beside truncation could be measured for MDHM. However, a so far not confirmed acetylation at K239 for MDHM needs to be further enlightened, as a possible relation between this modification and protein truncation could be closely related. A well-known phosphorylation for MLRV, responsible for force generation, was confirmed. In addition, an N-terminal cleaved MLRV species was measured. This species is shown for the first time to occur in non-diseased conditions. Our result demonstrated that for identification standard ESI–MS and FT-ICR-MS should be considered as complementary approaches enabling characterization of different modifications. The newly detected protein species were regulated in the myocardium of mice related to age, sex and oral genistein treatment. Therefore, those species could be relevant in cardiac disease and should be taken into consideration for the molecular understanding of pathological processes.

Acknowledgments This work was financially supported in part by the Hypatia Programme for young female scientists, Berlin and The Charite Grant for promotion of young academics, Berlin, both were awarded to K. Schwab.

References

Arrell DK, Neverova I, Van Eyk JE (2001) Cardiovascular proteomics: evolution and potential. Circ Res 88:763–773

Ashrafian H, Frenneaux MP, Opie LH (2007) Metabolic mechanisms in heart failure. Circulation 116:434–448

Baker L, Meldrum KK, Wang MJ, Sankula R, Vanam R, Raiesdana A, Tsai B, Hile K, Brown JW, Meldrum DR (2003) The role of estrogen in cardiovascular disease. J Surg Res 115:325–344

Casey TM, Arthur PG, Bogoyevitch MA (2005) Proteomic analysis reveals different protein changes during endothelin-1- or leukemic inhibitory factor-induced hypertrophy of cardiomyocytes in vitro. Mol Cell Proteomics 4:651–661

de Kleijn MJ, van der Schouw YT, Wilson PW, Grobbee DE, Jacques PF (2002) Dietary intake of phytoestrogens is associated with a favorable metabolic cardiovascular risk profile in postmenopausal U.S. women: the Framingham study. J Nutr 132:276–282

de Tombe PP (2003) Cardiac myofilaments: mechanics and regulation. J Biomech 36:721–730

Dello SP, Rovida E (2002) Transmodulation of cell surface regulatory molecules via ectodomain shedding. Biol Chem 383:69–83

Eckerskorn C, Strahler J, Hanash S, Lottspeich F (1988) Identification and characterization of proteins after two-dimensional electrophoresis by microsequence analysis demonstrated with leukemia protein markers. Electrophoresis 9:624

Foster DB, Van Eyk JE (1999) In search of the proteins that cause myocardial stunning. Circ Res 85:470–472

Hammer E, Phong TQ, Steil L, Klingel K, Salazar MG, Bernhardt J, Kandolf R, Kroemer HK, Felix SB, Völker U (2010) Viral myocarditis induced by Coxsackievirus B3 in A.BY/SnJ mice: analysis of changes in the myocardial proteome. Proteomics 10:1802–1818

Heukeshoven J, Dernick R (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6:103–112

Hodis HN (2008) Assessing benefits and risks of hormone therapy in 2008: new evidence, especially with regard to the heart. Cleve Clin J Med 75(Suppl 4):S3–S12

Hoehenwarter W, Ackermann R, Zimny-Arndt U, Kumar NM, Jungblut PR (2006) The necessity of functional proteomics: protein species and molecular function elucidation exemplified by in vivo alpha A crystallin N-terminal truncation. Amino Acids 31:317–323

Jäger D, Jungblut PR, Müller-Werdan U (2002) Separation and identification of human heart proteins. J Chromatogr B 771:131–153

Jungblut P, Thiede B, Zimny-Arndt U, Muller EC, Scheler C, Wittmann-Liebold B, Otto A (1996) Resolution power of two-dimensional electrophoresis and identification of proteins from gels. Electrophoresis 17:839–847

Jungblut PR, Holzhutter HG, Apweiler R, Schluter H (2008) The speciation of the proteome. Chem Cent J 2:16

Kao HJ, Cheng CF, Chen YH, Hung SI, Huang CC, Millington D, Kikuchi T, Wu JY, Chen YT (2006) ENU mutagenesis identifies mice with cardiac fibrosis and hepatic steatosis caused by a mutation in the mitochondrial trifunctional protein beta-subunit. Hum Mol Genet 15:3569–3577

Kim SC, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, Grishin NV, White M, Yang XJ, Zhao Y (2006) Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol Cell 23:607–618

Klose J, Kobalz U (1995) 2-Dimensional electrophoresis of proteins—an updated protocol and implications for a functional-analysis of the genome. Electrophoresis 16:1034–1059

Knecht M, Regitz-Zagrosek V, Pleissner KP, Emig S, Jungblut P, Hildebrandt A, Fleck E (1994) Dilated cardiomyopathy: computer-assisted analysis of endomyocardial biopsy protein patterns by two-dimensional gel electrophoresis. Eur J Clin Chem Clin Biochem 32:615–624

Kondo K, Suzuki Y, Ikeda Y, Umemura K (2002) Genistein, an isoflavone included in soy, inhibits thrombotic vessel occlusion in the mouse femoral artery and in vitro platelet aggregation. Eur J Pharmacol 455:53–57

Kuznetsov AV, Hermann M, Saks V, Hengster P, Margreiter R (2009) The cell-type specificity of mitochondrial dynamics. Int J Biochem Cell Biol 41:1928–1939

Lekgabe ED, Royce SG, Hewitson TD, Tang MLK, Zhao CX, Moore XL, Tregear GW, Bathgate RAD, Du XJ, Samuel CS (2006) The effects of relaxin and estrogen deficiency on collagen deposition and hypertrophy of nonreproductive organs. Endocrinology 147:5575–5583

McCarty MF (2006) Isoflavones made simple—Genistein's agonist activity for the beta-type estrogen receptor mediates their health benefits. Med Hypotheses 66:1093–1114

Meng C, Jin X, Xia L, Shen SM, Wang XL, Cai J, Chen GQ, Wang LS, Fang NY (2009) Alterations of mitochondrial enzymes contribute to cardiac hypertrophy before hypertension development in spontaneously hypertensive rats. J Proteome Res 8:2463–2475

Minarik P, Tomaskova N, Kollarova M, Antalik M (2002) Malate dehydrogenases—structure and function. Gen Physiol Biophys 21:257–265

Okkels LM, Muller EC, Schmid M, Rosenkrands I, Kaufmann SH, Andersen P, Jungblut PR (2004) CFP10 discriminates between nonacetylated and acetylated ESAT-6 of *Mycobacterium tuberculosis* by differential interaction. Proteomics 4:2954–2960

Rottbauer W, Wessels G, Dahme T, Just S, Trano N, Hassel D, Burns CG, Katus HA, Fishman MC (2006) Cardiac myosin light chain-2—a novel essential component of thick-myofilament assembly and contractility of the heart. Circ Res 99:323–331

Schluter H, Apweiler R, Holzhutter HG, Jungblut PR (2009) Finding one's way in proteomics: a protein species nomenclature. Chem Cent J 3:11

Schott P, Asif AR, Graf C, Toischer K, Hasenfuss G, Kogler H (2008) Myocardial adaptation of energy metabolism to elevated preload depends on calcineurin activity: a proteomic approach. Basic Res Cardiol 103:232–243

Song J, Kost CK, Martin DS (2006) Androgens augment renal vascular responses to ANG II in New Zealand genetically hypertensive rats. Am J Physiol Regul Integr Comp Physiol 290:R1608–R1615

Spiekerkoetter U, Sun B, Khuchua Z, Bennett MJ, Strauss AW (2003) Molecular and phenotypic heterogeneity in mitochondrial trifunctional protein deficiency due to beta-subunit mutations. Hum Mutat 21:598–607

Spiekerkoetter U, Khuchua Z, Yue Z, Bennett MJ, Strauss AW (2004) General mitochondrial trifunctional protein (TFP) deficiency as a result of either alpha- or beta-subunit mutations exhibits similar phenotypes because mutations in either subunit alter TFP complex expression and subunit turnover. Pediatr Res 55:190–196

Squadrito F, Altavilla D, Crisafulli A, Saitta A, Cucinotta D, Morabito N, D'Anna R, Corrado F, Ruggeri P, Frisina N, Squadrito G (2003) Effect of genistein on endothelial function in postmenopausal women: a randomized, double-blind, controlled study. Am J Med 114:470–476

Stanley WC, Recchia FA, Lopaschuk GD (2005) Myocardial substrate metabolism in the normal and failing heart. Physiol Rev 85:1093–1129

Vosseller K, Hansen KC, Chalkley RJ, Trinidad JC, Wells L, Hart GW, Burlingame AL (2005) Quantitative analysis of both protein expression and serine/threonine post-translational modifications through stable isotope labeling with dithiothreitol. Proteomics 5:388–398

Walker LA, Walker JS, Ambler SK, Buttrick PM (2009) Stage-specific changes in myofilament protein phosphorylation following myocardial infarction in mice. J Mol Cell Cardiol 48:1180–1186

White MY, Cordwell SJ, McCarron HC, Tchen AS, Hambly BD, Jeremy RW (2003) Modifications of myosin-regulatory light chain correlate with function of stunned myocardium. J Mol Cell Cardiol 35:833–840

Yotov WV, St Arnaud R (1993) Cloning and functional expression analysis of the alpha subunit of mouse ATP synthase. Biochem Biophys Res Commun 191:142–148

Zimny-Arndt U, Schmid M, Ackermann R, Jungblut PR (2009) Classical proteomics: two-dimensional electrophoresis/MALDI mass spectrometry. Methods Mol Biol 492:65–91