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Sex differences in murine myocardium are not exclusively regulated by gonadal hormones

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ABSTRACT

We investigated sex differences in cardiac protein patterns of intact and castrated mice using proteomics and 1D and 2D immunoblotting. To exclude differences concerning developmental aspects gonadectomy was conducted in mature mice at the age of three months. The main sex-related regulation in the protein pattern of the myocardium occurred for proteins involved in metabolic processes whereas only few proteins involved in other pathways underwent a regulation. Many regulated proteins (2/3) displayed a characteristic V form, which means that these proteins are up- or down-regulated in sexually mature compared to young mice and are back-regulated after castration, emphasizing a direct regulation by gonadal hormones. Several other spots (1/3) showed the same male/female regulation or a drastic increase in male/female spot intensity ratio after castration, suggesting either a regulation independent of sex hormones or a removal of an inhibiting feedback mechanism by gonadectomy. Technically, we found that it cannot be expected that a single spot contains only one protein species and that one protein is present in only one spot. We thus propose for proteomic investigations to identify/quantify all spots of a 2-DE pattern to obtain information about protein speciation and its potential importance for function and pathology.

Biological significance: Sex related differences in cardiovascular disease, including risk factors, disease manifestation and outcomes, are far from being well understood, and improved biological understanding of these differences in the healthy myocardium is of great importance. We investigated sex related changes of myocardial protein pattern in intact and castrated mice at different ages and found metabolic proteins to be highly regulated, some of which independently from gonadal hormones.

1. Introduction

Cardiovascular disease (CVD) is a worldwide continuously growing health concern, accounting for more than 50% of global deaths [1]. Generally, there is a greater and earlier manifestation of CVD in men compared to premenopausal women [2] but the cardiovascular risk for women rises after menopause [3]. Conflicting findings from randomized hormone replacement trials led to a controversy over protective actions of estrogens [4] and improved biological understanding of sex-related differences is of great importance to ameliorate diagnosis and treatment of CVD in the future [1].

There is an increasing number of studies reporting sex differences in the cardiovasculature [5]. Several genetic and experimental rodent models have been used to anlayze these aspects, e.g. models of ischemic injury, pressure overload and atherosclerosis (for summary see [6]) and most sex-related cardiac proteomics studies conducted in rodents use those disease models [7–9]. Only a few proteomics reports analyze the healthy myocardium, for example by mapping the murine cardiac proteome [10–12] or by studying age-related aspects in the rodent heart [13,14]. Cases in which sex differences in the healthy heart have been studied are rare [15–18].

In our study, we characterized sex-related protein differences in the non-diseased myocardium of intact mice and under hormone deprivation and for some proteins we did so at the protein species level. In the last two decades, it has become clear that the view on proteins has to be extended to the protein species level [19–21]. After synthesis at the

Abbreviations: CVD, cardiovascular disease; FA, fatty acid; ECHA, trifunctional enzyme subunit alpha; ECHB, trifunctional enzyme subunit beta; THIM, 3-ketoacyl-CoA thiolase; ER, estrogen receptor; PPARA, peroxisome proliferator-activated receptor alpha; PRGC1, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PTM, post-translational modification; Q-RT-PCR, quantitative real time PCR; RF, regulation factor; 1D-IB, one dimensional immunoblotting; 2D-IB, two dimensional immunoblotting

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ribosome, the primary protein species is chemically modified by post-translational modifications including addition of residues such as phosphates, methyl or acetyl groups and many others, truncations or even protein splicing processes. This results for each protein in the production of a huge diversity of protein species, each with a different chemical composition and a potentially different biological function. After protein synthesis there is an additional regulation level, which is ignored by the conventional bottom-up LC-MS techniques. Many phosphorylated peptides may be accessed [22], but the measurement of phosphorylated peptides completely ignores the fact that the combination of different PTMs determines the function and the combination of PTMs, represented in each protein species, is already destroyed by tryptic digestion before the MS identification. 2-DE-MS is a powerful tool to recognize protein speciation [23,24] but complete primary structure elucidation was obtained only in rare cases [25]. However, this complete characterization is necessary to understand which protein species is responsible for a certain function of a protein.

In the current work, we observed protein speciation for some proteins. Though a complete primary structure characterization of single protein species was not reached, even by the use of Orbitrap MS technology, our findings show that protein species separation before MS should be aspired and high-resolution 2-DE is the key method to reach the protein species level. Our differential proteomics analysis revealed mainly metabolic proteins to undergo a regulation in a sex-dependent manner, and among them especially proteins involved in fatty acid (FA) metabolism and mitochondrial function. Differential cardiac energetic between male and female mice was seen regardless of gonadectomy and this might contribute, at least in part, to the cardioprotective effect of the female sex.

2. Materials and methods

2.1. Animals and diets

Male and female C57BL/6J mice were maintained on basic, phytoestrogen free chow (2014S Teklad, Harlan, Germany) and had free access to food and water. Six animal groups, each comprising 10 mice, were included in this study: i) intact male mice, aged 1 month, ii) intact female mice, aged 1 month, iii) intact male mice, aged 6 month, iv) intact female mice, aged 6 month, v) castrated male mice, aged 6 month and vi) castrated female mice, aged 6 month. Gonadectomy was conducted in mice, aged 3 months, following standard procedures [26,27]. The relatively late time point for gonadectomy was chosen to allow the investigation of cardiac function in mature mice, while avoiding any development-related aspects. Animals (n = 10 per group) were sacrificed by cervical dislocation, hearts excised, washed thoroughly and were either snap-frozen in liquid nitrogen or fixed in formalin for histological analysis. Animal's body and heart weights were recorded and were expressed as group mean with SEM (standard error of the mean). All animal experiments were performed in accordance with the German Guidelines for the care and use of laboratory animals.

2.2. Urea protein extraction

Briefly, crushed frozen tissue was incubated for 45 min in 6 volumes of 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 16,000 × g at RT. To avoid carbamylation, ultrapure urea (#161-0731 from Biorad, Germany) was used. The supernatant was transferred to new tubes and the protein concentration was determined with the Bradford Reagent (Carl Roth, Germany).

2.3. 2-DE and comparative image analysis

Large-scale 2-DE (23 cm × 30 cm × 0.75 mm) and image analyses were conducted as described before [28]. Seven mice per group were analysed by 2-DE. For analytical gels 100 μg protein extract was loaded at the anodic side of IEF gels, focused at 8500Vh in a gradient between pI 2 and 11 and further separated in 15% acrylamide gels in Tris-glycine buffer (25 mM Tris and 192 mM glycine). Gels were fixed in 50% ethanol 10% acetic acid overnight and silver-stained according to Heukeshoven and Dernick [29]. Image analysis was performed with the Proteomweaver software version 3.0.9.9 (Biorad, Germany) according to the manufacturer's instructions. Briefly, spots were detected, their intensities normalized to total intensity of all spots (on one gel) and matching was carried out first inside groups and then between groups. Spot were edited and matched manually to reduce inaccurate spot matching. They were considered to be regulated when the relative spot density was significantly different ($p < 0.05$) with a regulation factor (RF) following the minimal significant factor and the considered spot present in at least five out of the seven gels in each study group. No further cut-off was applied and all protein spots with the above-described behavior were subjected to MS analysis.

2.4. ESI-MS/MS for 2-DE spot identification

All spots with significant change in amount were excised from preparative gels (23 cm × 30 cm × 1.5 mm, stained according to Nebrich [30]), in-gel digested with 200 ng trypsin per spot and tryptic peptides were subjected to nanoLC-ESI-MS/MS. The 2-DE spot positions and their peak lists (mgf files) are accessible via https://transfer.mpiib-berlin.mpg.de/index.php/s/dVzcs3VU3Of8ZFm, using the password murine.mgf.

The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Germany), PicoTip emitter (New Objective, USA) and an Esquire 3000 plus ion trap MS (Bruker, Germany). Peptide separation and MS criteria are described in details [28]. Peak lists were generated by DataAnalysis 3.2 (Bruker, Germany) with fragments qualified by amino acids, a minimal intensity of 25,000 and a maximum of 300 spectra.

Proteins were identified using MS/MS ion search of Mascot search engine Version No. 2.2 (www.matrixscience.com, Matrix Science, England) and the SwissProt data base (SwissProt 2010_07, Swiss Institute of Bioinformatics, Switzerland) with 16,299 sequences for *Mus musculus*. Parameters for data base searching were: *Mus musculus*, type of search (MS/MS Ion Search), enzyme (trypsin), variable modifications (oxidation M, propionamide C), mass values (monoisotopic), peptide mass tolerance (± 0.1%), fragment mass tolerance (± 0.5 Da) and maximal missed cleavages (1). Proteins with a significant score (significance threshold $p < 0.05$) and a minimum of 2 matched peptides have been considered to be properly identified. The pathway analysis tools Ingenuity Pathway Analysis (IPA, version 7.5, Ingenuity Systems, USA) was further used to identify prominent pathways altered due to conditions used in this study.

2.5. 1Dimensional immunoblotting (1D-IB)

20 μg urea protein extracts were separated in 10% acrylamide gels and transferred at low voltage overnight to a PVDF membrane in Towbin buffer, containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol [31], at 4 °C by semi-dry blotting following standard procedure [32]. The next day, membranes were blocked for 1 h in blocking solution (4% (w/v) BSA in TBS with 0.2% (v/v) Tween-20), incubated overnight at 4 °C in primary antibody (provider and dilutions, see below), diluted in blocking solution, washed 3 times in TBS-T and incubated for 1 h in secondary antibody (Dako, Denmark), diluted 1:5000 in blocking solution. After washing additional 3 times in TBS-T, membranes were overlaid with ECL solution (GE Healthcare, USA) and chemiluminescent signals were detected on hyper-films (GE Healthcare, USA). Antibodies against ER alpha (sc-544, diluted 1:500), ECHA (sc-292195, diluted 1:1000), ECHB (sc-55661, diluted 1:1000), THIM (sc-