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Differential proteomics reveals S100-A11 as a key factor in aldosterone-induced collagen expression in human cardiac fibroblasts

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ABSTRACT

Aldosterone (Aldo) could induce cardiac fibrosis, a hallmark of heart disease. Aldo direct effects on collagen production in cardiac fibroblasts remain controversial. Our aim is to characterize changes in the proteome of adult human cardiac fibroblasts treated with Aldo to identify new proteins altered that might be new therapeutic targets in cardiovascular diseases. Aldo increased collagens expressions in human cardiac fibroblasts. Complementary, using a quantitative proteomic approach, 30 proteins were found differentially expressed between control and Aldo-treated cardiac fibroblasts. Among these proteins, 7 were up-regulated and 23 were down-regulated by Aldo. From the up-regulated proteins, collagen type I, collagen type III, collagen type VI and S100-A11 were verified by Western blot. Moreover, protein interaction networks revealed a functional link between a third of Aldo-modulated proteome and specific survival routes. S100-A11 was identified as a possible link between Aldo and collagen. Interestingly, CRISPR/Cas9-mediated knock-down of S100-A11 blocked Aldo-induced collagen production in human cardiac fibroblasts. In adult human cardiac fibroblasts treated with Aldo, proteomic analyses revealed an increase in collagen production. S100-A11 was identified as a new regulator of Aldo-induced collagen production in human cardiac fibroblasts. These data could identify new candidate proteins for the treatment of cardiac fibrosis in cardiovascular diseases.

Significance: S100-A11 is identified by a proteomic approach as a novel regulator of Aldosterone-induced collagen production in human cardiac fibroblasts. Our data could identify new candidate proteins of interest for the treatment of cardiac fibrosis in cardiovascular diseases.

1. Introduction

Aldosterone (Aldo), a mineralocorticoid hormone that acts classically via an intracellular mineralocorticoid receptor (MR), is a well-known key regulator of blood pressure and electrolytic balance [1]. A growing body of clinical and pre-clinical evidence suggests that Aldo plays an important pathophysiological role in cardiac remodeling by promoting changes involving cardiac fibrosis [2]. Evidences from animal experiments plus the large randomized controlled trials RALES, EPHESUS and EMPHASIS studies in patients with heart failure (HF) suggest that chronic MR blockade reduces markedly the biological markers of cardiac fibrosis, suggesting that Aldo is an important determinant of cardiac collagen turnover [3–6]. In vitro, the literature concerning the effects of Aldo on collagen expression in cardiac fibroblasts is controversial. On one hand, it has been published that Aldo increases collagen synthesis in rat cardiac fibroblasts [7,8]. On the other hand, no differences in collagen expression upon Aldo stimulation were found in other studies using rat cardiac fibroblasts [9–11]. Moreover, it has been recently published that Aldo could contribute to collagen accumulation by inducing tissue inhibitor of metalloproteinases-1 (TIMP-1) expression, without modifying collagen mRNA expressions in human cardiac fibroblasts obtained from fetal hearts [12]. Thus, it is uncertain whether Aldo affects cardiac fibrosis in vivo by a direct effect on cardiac fibroblasts or an indirect mechanism.

The whole picture of Aldo effects on collagen production in cardiac fibroblasts is still unclear. The aim of this study, therefore, is to analyze direct Aldo effects on adult cardiac fibroblasts, an in vitro model isolated from human hearts. A proteomic approach has been used for the characterization of the proteostasis impairment after Aldo treatment in order to establish the protein interactome network regulated by Aldo. A better knowledge of the underlying mechanisms of Aldo effects on adult

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human cardiac fibroblasts may highlight novel mediators of cardiac fibrosis which could identify new biotargets for pharmacological therapies.

2. Methods

2.1. Cell culture

Adult human cardiac fibroblasts were obtained from Promocell and maintained in medium Fibroblasts Media 3. All assays in the present study were done at temperatures of 37 °C, 95% sterile air and 5% CO_2 in a saturation humidified incubator. Cells were used between passages 5 and 7. For experiments, cells were seeded into 6-well plates at 90% confluence and serum starved for 12 h. Then, cells were cultured in the same medium and stimulated with Aldo (10^{-8} M, Sigma) for 24 h for proteome exploration and for 5, 10, 30 and 60 min for intracellular pathways analysis.

2.2. Mass spectrometry-based quantitative proteomics

2.2.1. Sample preparation for proteomic analysis

Cellular pellets derived from untreated cardiac fibroblasts and Aldo-treated cardiac fibroblasts were resuspended in lysis buffer containing 7 M urea, 2 M thiourea, 4% (v/v) CHAPS, 50 mM DTT. Homogenates were spinned down at 14,000 rpm for 1 h at 15 °C. Protein concentration was measured in the supernatants with the Bradford assay kit (Bio-rad).

2.2.2. Proteomic analysis using iTRAQ approach

A shotgun comparative proteomic analysis of total cell extracts using iTRAQ (isobaric Tags for Relative and Absolute Quantitation) was performed [13]. Global experiments were carried out with three biological replicates in each experimental condition.

2.2.3. Peptide labeling

Protein extracts (160 μg) were precipitated with methanol/chloroform, and pellets dissolved in 7 M urea, 2 M thiourea, 4% (v/v) CHAPS. Protein quantitation was performed with the Bradford assay kit (Bio-Rad). iTRAQ labeling of each sample was performed according to the manufacturer's protocol (Sciex). Briefly, a total of 80 μg of protein from each cellular condition was reduced with 50 mM tris (2-carboxyethyl) phosphine (TCEP) at 60 °C for 1 h, and cysteine residues were alkylated with 200 mM methylmethanethiosulfonate (MMTS) at room temperature for 15 min. Protein enzymatic cleavage was carried out with trypsin (Promega; 1:20, w/w) at 37 °C for 16 h. Each tryptic digest was labelled according to the manufacturer's instructions with one isobaric amine-reactive tags as follows: Tag113, control cells-1; Tag114, control cells-2; Tag117, control cells-3; Tag118, Aldo-treated cells-1; Tag119, Aldo-treated cells-2; Tag121, Aldo-treated cells-3. After 1 h incubation, each set of labelled samples were independently pooled and evaporated until <40 μl in a vacuum centrifuge.

2.2.4. Peptide fractionation

To increase the proteome coverage, the peptide pool was injected to an Ettan LC system with a X-Terra RP18 pre-column (2.1 × 20 mm) and a high pH stable X-Terra RP18 column (C18; 2.1 mm × 150 mm; 3.5 μm) (Waters) at a flow rate of 40 μl/min. Peptides were eluted with a mobile phase B of 5–65% linear gradient over 35 min (A, 5 mM ammonium bicarbonate in water at pH 9.8; B, 5 mM ammonium bicarbonate in acetonitrile at pH 9.8). 6 fractions were collected, evaporated under vacuum and reconstituted into 20 μl of 2% acetonitrile, 0.1% formic acid, 98% MilliQ-H20 prior to mass spectrometric analysis.

2.2.5. Mass spectrometry analysis

Peptides mixtures were separated by reverse phase chromatography using an Eksigent nanoLC ultra 2D pump fitted with a 75 μm ID column (Eksigent 0.075 × 150). Samples were first loaded for desalting and concentration into a 0.5 cm length 300 μm ID pre-column packed with the same chemistry as the separating column. Mobile phases were 100% water 0.1% formic acid (FA) (buffer A) and 100% Acetonitrile 0.1% FA (buffer B). Column gradient was developed in a 70 min two step gradient from 2% B to 30% B in 60 min and 30%B to 40% B in 10 min. Column was equilibrated in 95% B for 5 min and 2% B for 15 min. During all process, pre-column was in line with column and flow maintained all along the gradient at 300 nl/min. Eluting peptides from the column were analyzed using an AB Sciex 5600 TripleTOF™ system. Information data acquisition was acquired upon a survey scan performed in a mass range from 350 *m/z* up to 1250 *m/z* in a scan time of 250 ms. Top 25 peaks were selected for fragmentation. Minimum accumulation time for MS/MS was set to 75 ms giving a total cycle time of 2.1 s. Product ions were scanned in a mass range from 100 *m/z* up to 1700 *m/z* and excluded for further fragmentation during 15 s. After MS/MS analysis, data files were processed using ProteinPilot™ 4.5 software from Sciex which uses the algorithm Paragon™ (v.4.0.0.0) [14] for database search and Progroup™ for data grouping and searched against Uniprot mouse database. False discovery rate was performed using a non-lineal fitting method and displayed results were those reporting a 1% Global False Discovery Rate (FDR) or better. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) [15] via the PRIDE partner repository with the data set identifiers PXD005879 (Username: reviewer92254@ebi.ac.uk; password: hnIlQv6N).

2.2.6. Data analysis

Relative quantification and protein identification were performed with the ProteinPilot™ software (version 4.5; Sciex) using the Paragon ™ algorithm as the search engine. Each MS/MS spectrum was searched against a database of human protein sequences (Uniprot Human Reference database, December 2015). The search parameters allowed for cysteine modification by MMTS and biological modifications program in the algorithm (i.e. phosphorylations, amidations, semitryptic fragments, etc.). Reporter ion intensities were bias corrected for the overlapping isotope contributions from the iTRAQ tags according to the certificate of analysis provided by the reagent manufacturer (Sciex). The peptide and protein selection criteria for relative quantitation were performed as follows. Only peptides unique for a given protein were considered for relative quantitation, excluding those common to other isoforms or proteins of the same family. Proteins were identified on the basis of having at least one peptide with an ion score above 99% confidence. Among the identified peptides, some of them were excluded from the quantitative analysis for one of the following reasons: (i) The peaks corresponding to the iTRAQ labels were not detected; (ii) the peptides were identified with low identification confidence (<1.0%); (iii) the sum of the signal-to-noise ratio for all of the peak pairs was <6 for the peptide ratios. The protein sequence coverage (95% conf.) was estimated for specific proteins by the percentage of matching amino acids from the identified peptides having confidence greater than or equal to 95% divided by the total number of amino acids in the sequence. Several quantitative estimates provided for each protein by ProteinPilot were utilized: the fold change ratios of differential expression between labelled protein extracts; the p-value, representing the probability that the observed ratio is different than 1 by chance. A decoy database search strategy was also used to estimate the false discovery rate (FDR), defined as the percentage of decoy proteins identified against the total protein identification. The FDR was calculated by searching the spectra against the decoy database generated from the target database. The results were then exported into Excel for manual data interpretation. Although relative quantification and statistical analysis were provided by the ProteinPilot software, an additional 1.3-fold change cutoff for all iTRAQ ratios (ratio < 0.77 or >1.3) and a p-value lower than 0.05 were selected to classify proteins as up- or down-regulated (at least in two of three biological replicates). Proteins with iTRAQ ratios below

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