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Original article

Quantitative temporal analysis of protein dynamics in cardiac remodeling

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ABSTRACT

Cardiac remodeling (CR) is a complex dynamic process common to many heart diseases. CR is characterized as a temporal progression of global adaptive and maladaptive perturbations. The complex nature of this process clouds a comprehensive understanding of CR, but greater insight into the processes and mechanisms has potential to identify new therapeutic targets. To provide a deeper understanding of this important cardiac process, we applied a new proteomic technique, PALM (Pulse Azidohomoalanine in Mammals), to quantitate the newly-synthesized protein (NSP) changes during the progression of isoproterenol (ISO)-induced CR in the mouse left ventricle. This analysis revealed a complex combination of adaptive and maladaptive alterations at acute and prolonged time points including the identification of proteins not previously associated with CR. We also combined the PALM dataset with our published protein turnover rate dataset to identify putative biochemical mechanisms underlying CR. The novel integration of analyzing NSPs together with their protein turnover rates demonstrated that alterations in specific biological pathways (e.g., inflammation and oxidative stress) are produced by differential regulation of protein synthesis and degradation.

1. Introduction

Heart failure (HF) and cardiac remodeling (CR) are both common stages of many heart diseases with increasing incidence and prevalence in the United States, posing major public health problems. CR is a multifactorial process and results not only from cardiac overload or injury but also from a complex interplay among genetic, inflammatory, biochemical, and neuro-hormonal alterations [46]. Accordingly, the CR process involves prolonged over-stimulation of the sympathetic nervous system (SNS) followed by the release of catecholamines to increase both the heart rate and individual cellular contractility, thereby creating a compensatory adaptive response that transiently normalizes the biomechanical stress and optimizes cardiac contractility. Catecholamines are crucial stress hormones and regulators of heart function via stimulation of beta-adrenergic receptors (β-ARs). Chronic over-stimulation, however, augments the CR process, eventually leading to cardiac dysfunction and HF [5, 27]. CR in the early stages exhibits thickening of the ventricular and interventricular walls and is characterized by increasing size of the cardiomyocytes, changes in the organization of the sarcomeric structure, and increased protein synthesis [15]. Similarly, persistent pharmacological β-AR stimulation with the agonist isoproterenol (ISO) can cause left ventricle hypertrophy and HF in laboratory animals [41, 50]. Numerous studies have suggested that chronic ISO application stimulates structural and metabolic remodeling, which alters fatty acid utilization, glucose homeostasis, extracellular matrix turnover and mitochondrial function ([10, 51]. To advance our knowledge in the underlying molecular mechanisms of CR and HF, it is important to identify how proteome alterations gradually manifest during these pathophysiological stages over time. Identification of proteins that drive CR may lead to novel therapeutic drug targets or clinical biomarkers for cardiac pathologies.

Quantitative proteomics has great potential to better understand the myriad of proteome alterations during the progression of ISO-induced CR. We recently developed the quantitative proteomic technique PALM (Pulse Azidohomoalanine Labeling in Mammals) to improve the temporal resolution of quantitative proteomics [30]. PALM quantitates newly-synthesized proteins (NSPs). Since the NSP sub-proteome in theory is the first to respond to perturbations, quantitation of NSPs is

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more sensitive than quantitation of the whole proteome. PALM relies on azidohomoalanine (AHA), which is a non-canonical amino acid that is accepted by the endogenous methionine tRNA and inserted into proteins in vivo [14]. AHA is inserted into the mouse proteome through a customized AHA diet. The AHA diet is given to mice within a discrete time period to label NSPs in response to a perturbation. Proteins incorporating AHA can be covalently linked in vitro to a biotin-alkyne with click chemistry. Through standard biotin enrichment strategies, the static or "old" proteome is removed, and the remaining NSPs can be identified and quantitated using mass spectrometry (MS). In this study, we applied the PALM strategy to the ISO-induced CR mouse model, in which we quantify newly synthesized proteins through an acute phase, during which the CR process is at its highest rate, and a prolonged phase, during which the CR process has reached a plateau, to enhance our mechanistic understanding of cardiac proteome remodeling in this pathophysiological process. In the normal heart, homeostasis of continuous protein synthesis and degradation is essential to maintain cardiac function at the cellular and whole-organ level [21]. Normal protein homeostasis through the protein turnover cycle is altered during CR and HF through factors such as hypertrophic signaling [46], calcium regulation [19], inflammatory reactions [46], and oxidative stress (Goirdano FJ et al., JCI 2005;115(3);500–508). For the first time, this study integrates two novel datasets using previously published protein turnover rates in the ISO-induced CR mouse model with the NSP quantitation in response to ISO treatment. This integrated analysis provides unique insight into the total balance of protein synthesis and degradation between two compared groups (i.e., sham group vs. ISO group) and highlights the mechanisms that underlie proteomic changes in CR and establishes a novel experimental and bioinformatic approach that is generally applicable to animal models of disease.

2. Methods

2.1. Animals/surgery/tissue collection

CR was induced by continuous ISO treatment for 14 days in male C57/BL6 wildtype mice (aged 8–12 weeks) using microosmotic pumps (Alzet, Model 1002). ISO was dissolved in phosphate-buffered saline 1× (PBS). The microosmotic pumps were implanted subcutaneously in the posterior neck area of the mouse under 2% isoflurane (vaporized in oxygen) anesthesia. One group of mice received continuous treatment of ISO with a dose of 15 mg/kg/day for a total period of 14 days. As a control, a second group of mice underwent sham treatment by vehicle (PBS).

AHA-Heavy pellets were fed to the ISO group and AHA-Light pellets to the control group. For efficient labeling as well as to study two different phases in the CR process, we collected samples on Day 4 and Day 14 in both the ISO group and the control group. To collect samples on Day 4, we commenced AHA labeling on Day 0. To collect samples on Day 14, we commenced AHA labeling on Day 10. To conduct our AHA labeling analyses after the 14-day period treatment, whole heart, skeletal muscle, liver, kidney, and brain were collected after sacrificing the mice. In contrast to sham treatment, the heart weight-body weight ratios (HW/BW) were increased in the ISO group on Day 4 and Day 14, confirming cardiac remodeling. Cardiac tissues were collected from left ventricle (LV) and right ventricle (RV) and rinsed in PBS. The septum was included as part of the left ventricle.

2.2. Assesment of mitochondrial function

Mitochondria were isolated from mouse hearts followed by mitochondrial electron transport chain (ETC) activity assays as previously described [12].

2.3. Tissue homogenization

LVs were transferred into Precellys CK14 tubes containing 1.4-mm ceramic beads, and 0.5-mm disruption beads (Research Products International Corp.) as well as 500-ul PBS were added. The homogenization was performed on Bertin Precellys bead-beating system: 6500 rpm, 3 times, 20 s internal. Then, the homogenates were sonicated for 30 s after adding 0.5% SDS. A BCA protein assay was performed on each sample.

2.4. Click chemistry

First, 2.5-mg protein of LV from heavy AHA (H-AHA)-labeled ISO mice or sham mice and 2.5-mg protein of LV from light AHA (L-AHA)-labeled sham mice or ISO mice were mixed together as one biological replicate. For each biological replicate, the H-AHA/L-AHA mixture was divided into twenty aliquots (0.25 mg/aliquot). A click reaction was performed on each aliquot as previously published [47]. Briefly, for each click reaction, the following reagents were added in this order: 1) 30 ul of 1.7-mM TBTA, 2) 8 ul of 50-mM Copper Sulfate, 3) 8 ul of 5-mM Biotin-Alkyne ($C_{21}H_{35}N_3O_6S$, Click Chemistry Tools), and 4) 8 ul of 50-mM TCEP. PBS was then added to a final volume of 400 ul and incubated for 1 h at room temperature. Methanol/Chloroform precipitation was performed, and the precipitated protein was combined so that there would only be one pellet per 5-mg starting material.

2.5. Digestion and biotin peptide enrichment

Biotin peptide enrichment was performed as previously described [44]. Briefly, precipitated pellets were resuspended in 100-ul 8 M urea and 100-ul 0.2% MS-compatible surfactant ProteaseMAX (Promega) in 50 mM ammonium bicarbonate, then reduced, alkylated, and digested with trypsin as previously described [44]. The digestion was then centrifuged at 13,000 ×*g* for 10 min. The supernatant was transferred to a new tube, and the pellet was resuspended with PBS and centrifuged at 13,000 ×*g* for 10 min. Supernatants were combined, and 150 ul of neutravidin agarose resin (Thermo Scientific) was added. The resin was incubated with the peptides for 2 h at room temperature while rotating. The resin was then washed with 1-ml PBS, then PBS with 5% acetonitrile, PBS, and a final wash of distilled water. The peptides were eluted two times off the resin with 150-ul 80% acetonitrile, 0.2% formic acid, and 0.1% TFA on shaker for 5 min at room temperature, and another two times on a shaker at 70 °C. All elutions were transferred to a single new tube. Prior to MS analysis, the samples were dried with a speed-vac, and dried peptides were re-solubilized in buffer A (5% ACN, 95% water, 0.1% formic acid).

2.6. Mass spectrometry

Soluble peptides were pressure-loaded onto a 250-μm i.d. capillary with a kasil frit containing 2.5 cm of 10-μm Jupiter C18-A material (Phenomenex) followed by 2.5 cm of 5-μm Partisphere strong cation exchanger (Whatman). This column was washed with buffer A after loading. A 100-μm i.d. capillary with a 5-μm pulled tip packed with 15 cm of 4-μm Jupiter C18 material (Phenomenex) was attached to the loading column with a union, and the entire split-column (loading column–union–analytical column) was placed in line with an Agilent 1100 quaternary HPLC (Palo Alto). The sample was analyzed using MudPIT, which is a modified 11-step separation described previously [55]. The buffer solutions used were buffer A, 80% acetonitrile/0.1% formic acid (buffer B), and 500-mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Step 1 consisted of a 10 min gradient from 0 to 10% buffer B, a 50 min gradient from 10 to 50% buffer B, a 10 min gradient from 50 to 100% buffer B, and 20 min from 100% buffer A. Steps 2 consisted of 1 min of 100% buffer A, 4 min of 20% buffer C, a 5-min gradient from 0 to 10% buffer B, an 80-min gradient