Experimental Gerontology 43 (2008) 909–918

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Differential protein expression during aging in ventricular myocardium of Fischer 344 × Brown Norway hybrid rats

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ARTICLE INFO

Article history:
Received 6 May 2008
Received in revised form 24 June 2008
Accepted 8 July 2008
Available online 15 July 2008

Keywords:
Aging
F344 × BN hybrid
heart
MALDI-TOF
Nitrosylation
Peptide mass fingerprinting
Proteomics
Two-dimensional electrophoresis

ABSTRACT

The aging heart undergoes well characterized structural changes associated with functional decline, though the underlying mechanisms are not understood. The aim of this study was to determine to what extent ventricular myocardial protein expression was altered with age and which proteins underwent protein nitration. Fischer 344 × Brown Norway F1 hybrid (FBN) rats of four age groups were used, 4, 12, 24, and 34 months. Differential protein expression was determined by 2-DE and proteins were identified by peptide mass fingerprinting. Altered protein nitration with age was assessed by immunoblotting. Over 1000 protein spots per sample were detected, and 255 were found to be differentially expressed when all aged groups were compared to young rats (4 months) ($p \leqslant 0.05$). A strong positive correlation between differential protein expression and increasing age ($p = 0.03$, $R^2 = 0.997$) indicated a progressive, rather than abrupt, change with age. Of 46 differentially expressed proteins identified, seventeen have roles in apoptosis, ten in hypertrophy, seven in fibrosis, and three in diastolic dysfunction, aging-associated processes previously reported in both human and FBN rat heart. Protein expression alterations detected here could have beneficial effects on cardiac function; thus, our data indicate a largely adaptive change in protein expression during aging. In contrast, differential protein nitration increased abruptly, rather than progressively, at 24 months of age. Altogether, the results suggest that differential myocardial protein expression occurs in a progressive manner during aging, and that a proteomic-based approach is an effective method for the identification of potential therapeutic targets to mitigate aging-related myocardial dysfunction.

1. Introduction

Aging is a major risk factor for developing disease, including cardiovascular disease and cancer (Lakatta and Levy, 2003). Thus, as the elderly percentage of the population rapidly increases in the near future, there will be a resultant rise in age-related cardiovascular disease. A major aspect of the aging process is a gradual decline in biological functions, and the human heart undergoes structural and functional changes with age, resulting in a decline in cardiac performance (Gates et al., 2003; Groban, 2005; Oxenham and Sharpe, 2003). While these systemic changes are well documented, the changes that occur at the cellular and molecular level are less well understood. Growing evidence implicates oxidative stress such as that caused by reactive nitrogen species (RNS) as a causal factor for cellular dysfunction and it is well accepted that oxidative stress increases with age (Barja, 2002; Sohal and Weindruch, 1996).

To provide a better understanding of the protein molecular machinery underlying the aging process, we employed a proteomic platform capable of characterizing changes in protein expression and post-translational modifications. Although many theories of aging have been proposed, such as cellular senescence (Hayflick, 1965), gene expression order (Helfand and Rogina, 2000), and free radical generation (Harman, 1981), the mechanism that accounts for biological aging is currently viewed as a complex, multifactorial process. Alterations in proteins also have been proposed to contribute to aging-related impairments (Levine and Stadtman, 2001), and tools of proteomics such as the field's cornerstone, two-dimensional electrophoresis (2-DE), are well suited to analyze complex quantitative and qualitative changes with age. This analysis of differential expression and modification yields a semi-global view of the underlying molecular changes accompanying physiological processes such as aging, which can assist in elucidating its mechanisms. Furthermore, these exploratory experiments are necessary to increase the likelihood of discovering novel proteins regulating aging phenomena and potential therapeutic targets for aging-related dysfunction.

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0531-5565/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.exger.2008.07.003

The majority of aging studies have compared only very young to extremely old animals. A primary goal of this study was to determine whether global and individual changes in ventricular myocardial protein expression during aging occurred in a progressive or in an abrupt manner. We also examined post-translational protein nitration (3-nitrotyrosine) of specific proteins as an index of age-related oxidative stress. To this end, hearts from Fischer 344 X Brown Norway hybrid (FNB) rats, ages 4, 12, 24, and 34 months were obtained from the NIA for analysis of differential protein expression and nitrotyrosine modification.

2. Materials and methods

2.1. Materials

IPG strips and acrylamide for slab gels were purchased from Bio-Rad (Richmond, CA). Other ultrapure electrophoretic reagents were obtained from Bio-Rad (Richmond, CA), Sigma Chemical Co. (St. Louis, MO), or BDH (Poole, UK). Sequence grade trypsin was obtained from Promega (Madison, WI). Ammonium bicarbonate was purchased from Mallinckrodt Chemicals (Paris, KY). Formic acid, iodoethanol, and triethylphosphine were obtained from Sigma–Aldrich Co. (St. Louis, MO). Acetonitrile and hydrochloric acid solution N/10 were obtained from Fisher Scientific (Fair Lawn, NJ). PVDF for Western blotting was obtained from Millipore Co. (Billerica, MA). All other chemicals used were of the highest grade obtainable.

2.2. Tissues

Male Fischer 344 X Brown Norway hybrid (FBN) rat hearts (ages 4, 12, 24, and 34 months; $n = 5$) were purchased from the National Institute of Aging (NIA). The rodent colonies were barrier maintained and kept Specific Pathogen Free (SPF). The rats were euthanized by CO_2 asphyxiation and their hearts were removed, flash frozen in liquid N_2, and stored at −80 °C. The hearts were shipped on dry ice and stored at −45 °C immediately upon arrival.

2.3. Preparation of ventricular myocardium (VM)

On ice, 150 mg of tissue was cut from the apex from each heart (VM) and massaged in ice cold filtered saline to remove as much blood as possible. The VM was then thoroughly minced with surgical scissors in a 50 ml beaker along with eight volumes of a solution containing 9 M urea, 4% Igepal CA-630 ([octylphenoxy] polyethoxyethanol), 1% DTT and 2% carrier ampholytes (pH 3–10). The minced samples were then placed in 3 ml DUALL® ground-glass tissue grinders and manually homogenized. To complete the solubilization, the minced/ground samples were sonicated with 3 × 2 s bursts at instrument setting three using a Microson Ultrasonic Cell Disruptor (Misonix, Inc.). This was repeated every 15 min for 1 h at room temperature. After complete solubilization at room temperature for 120 min, samples were centrifuged at 50,000 rpm at 15 °C for 20 min using a Beckman TL-100 ultracentrifuge to remove nucleic acid and insoluble materials, and the supernates stored at −45 °C until 2-DE separation.

2.4. Two-dimensional electrophoresis and image analysis

Reagent Compatible Detergent Compatible (RC DC) protein concentration assays were performed for each of the 20 samples using an RC DC Protein Assay Kit according to the manufacturer's protocol (Bio-Rad). Aliquots (~20–50 μl each) containing 400 μg of protein from each sample were diluted with ~450–480 μl rehydration buffer (8 M urea, 2% CHAPS, 15 mM DTT, 0.2% ampholytes pH 3–10, and 0.001% orange G). The resulting 500 μl protein dilutions were loaded onto IPG strips (24 cm, linear pH 3–10) by overnight, passive rehydration at room temperature. Isoelectric focusing was performed simultaneously on all IPG strips using two Protean IEF Cells (Bio-Rad), by a program of progressively increasing voltage (150 V for 2 h, 300 V for 4 h, 1500 V for 1 h, 5000 V for 5 h, 7000 V for 6 h, and 10,000 V for 3 h) for a total of 100,000 Vh. A computer-controlled gradient casting system was used to prepare the second-dimension SDS gradient slab gels (20 × 25 × 0.15 cm) in which the acrylamide concentration varied linearly from 11% to 17% T. First-dimension IPG strips were loaded directly onto the slab gels following equilibration for 10 min in Equilibration Buffer I and 10 min in Equilibration Buffer II (Equilibration Buffer I: 6 M urea, 2% SDS, 0.375 M Tris–HCl pH 8.8, 20% Glycerol, 130 mM DTT; Equilibration Buffer II: 6 M urea, 2% SDS, 0.375 M Tris–HCl pH 8.8, 20% Glycerol, 135 mM iodoacetamide) for alkylation and reduction of proteins. Second-dimension slab gels were run in parallel at 8 °C for 18 h at 160 V. Slab gels were stained using a colloidal Coomassie Blue G-250 procedure (Candiano et al., 2004). Gels were fixed in 1.5 L/10 gels of 50% ethanol/2% phosphoric acid overnight followed by three 30 min washes in 2 L/10 gels of deionized water. Gels were transferred to 1.5 L/10 gels of 30% methanol/17% ammonium sulfate/3% phosphoric acid for 1 h followed by addition of 1.2 g/L of powdered Coomassie Blue G-250 stain. After 96 h, gels were washed several times with water and scanned at 95.3 μm/pixel resolution using a GS-800 Calibrated Imaging Densitometer (Bio-Rad, Hercules CA). The resulting 12 bit images were analyzed using PDQuest™ software (Bio-Rad, v.7.1). Background was subtracted and peaks for the protein spots located and counted. Each gel image was normalized against the total gel density. PDQuest™ performs a preliminary statistical analysis using Student's *T*-test to compare groups and determine differential expression candidates. Sigma Stat 3.0 (Systat Software, Inc., San Jose, CA) was used to calculate the regression coefficient when comparing changes in the number of these candidates with age. PDQuest™ data was exported to Sigma Stat 3.0 for 1-way analysis of variance (ANOVA). Proteins were selected for MS identification based on these calculations.

2.5. Post-translational modification analysis via Western blotting

Immediately upon completion of a second, replicate 2-DE run, gels from all four groups ($n = 3$) were carefully removed and equilibrated in transfer buffer (49 mM Tris, 39 mM Glycine, 10% SDS, 20% MeOH, pH 9.2) for 1 h at room temperature. The PVDF membrane was immersed briefly in 100% methanol and then transfer buffer before being placed on the transfer soaked filter paper in the Transblot® SD Semi-Dry Transfer Cell (Bio-Rad). Equilibrated gels were placed directly on top of the PVDF membrane. A maximum of two gels were stacked in the Semi-Dry Transfer Cells and each Cell was connected to a separate Power Pac HC (Bio-Rad). Power supplies were set at 25 V and protein spots were transferred to PVDF paper over the course of 2 h. Following transfer, the gels were removed and stained as described above to evaluate transfer efficiency. The PVDF membranes were removed from the Transfer Cells and allowed to dry completely. Each membrane was then blocked with PBS-0.3% Tween20 for 2 h using 3–5 washes at room temperature and then incubated overnight with a primary anti-3-nitrotyrosine antibody (clone 1A6, Upstate/Millipore, Billerica, MA), diluted 1:10,000 in PBS-0.1% Tween20. The unbound antibody was rinsed off with 5–6 changes of PBS-0.1% Tween20 in 30–45 min. The secondary antibody (Goat Anti-mouse IgG, HRP conjugate, Upstate/Millipore, Billerica, MA) was diluted 1:10,000 in PBS-0.1% Tween20 and incubated for 2 h at room temperature. The unbound antibody was rinsed off with 5–6 changes of PBS-0.1% Tween20 in 30–45 min. The membranes are then developed in 10–15 ml of tetramethylbenzidine (TMB) for exactly