Journal of Molecular and Cellular Cardiology 113 (2017) 1–8

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Ascorbate starvation alters endoplasmic reticulum-resident enzymes in cardiac fibroblasts, priming them for increased procollagen secretion

Randy T. Cowling*, Joong Il Park[1], Ayodeji E. Sotimehin[2], Barry H. Greenberg

Department of Medicine, Division of Cardiovascular Medicine, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093, United States

ARTICLE INFO

Keywords:
Collagen
Prolyl 4-hydroxylase
Endoplasmic reticulum
Fibrosis
Secretion
Ascorbic acid

ABSTRACT

Since ascorbate is unnecessary for cell growth and survival, cardiac fibroblasts are routinely cultured without it. However, ascorbate is necessary for optimal collagen synthesis, so we hypothesized that its presence would influence cell phenotype.

Cardiac fibroblasts cultured without ascorbate had increased intracellular levels of procollagens, with procollagen α1(III) showing the largest accumulation. Endoplasmic reticulum (ER)-resident proteins that are known to bind single-stranded procollagens were also elevated. These included the catalytic prolyl 4-hydroxylase subunits, lysyl hydroxylases, and hydroxylysyl galactosyltransferases, with prolyl 4-hydroxylase α1 and α2 (P4HA1 and P4HA2) demonstrating the largest increases. There were no differences in the levels of protein disulfide isomerase (P4HB/PDI) or the triple-helical procollagen chaperone, HSP47, with or without ascorbate. Results were similar with mouse and rat cardiac fibroblasts, suggesting a conserved response. Ascorbate-replete cells that were subsequently deprived of the vitamin lost the ability to secrete intact procollagen α1(I) within ~3 days, approximately when intracellular procollagen α1(III) and P4HA1 levels began to rise. Upon ascorbate re-addition, starved fibroblasts initially secreted high levels of procollagen that gradually declined over ~4 days, a pattern that was not universal as extra domain A (EDA)-fibronectin secretion was unchanged. Despite the necessity of the P4HA enzymes for triple-helical procollagen formation, they were not responsible for early increased secretion. However, in the absence of ascorbate, P4HA2 overexpression increased intracellular turnover of procollagens, suggesting that it may help clear accumulating procollagens from the ER.

Cardiac fibroblasts change in the absence of ascorbate to cope with increased intracellular levels of procollagens. These changes occur slowly and can render the cells phenotypically altered for several days after ascorbate re-addition. These findings have direct implications for the study of cardiac fibroblasts in culture, and may help our understanding of the response of these cells to fluctuating nutrient levels in ischemic myocardium.

1. Introduction

Cardiac fibroblasts are mesenchymal cells found in the myocardial interstitium that are responsible for deposition and maintenance of non-vascular collagen in both healthy and diseased hearts. They have been estimated to represent 10–20% of cell numbers in mouse hearts [1], ~64% in rat hearts [2], and 43–58% in human hearts [3]. Many factors have been shown to activate cardiac fibroblasts and upregulate expression of collagen genes, including angiotensin II [4], transforming growth factor-β1 [5,6], platelet-derived growth factor [7], and endothelin-1 [8]. Although most studies of collagen deposition in the heart have focused on gene and/or protein expression, collagen production is a complicated, multi-step process that involves more than just transcription and translation of collagen genes.

After translation into the endoplasmic reticulum (ER), single-stranded procollagens are modified by prolyl hydroxylation, lysyl hydroxylation, and subsequent hydroxylysyl glycosylation. There are 3 known catalytic subunits of prolyl 4-hydroxylase (P4HA1, P4HA2, and P4HA3), 3 lysyl hydroxylases (PLOD1, PLOD2, and PLOD3, also called LH1, LH2, and LH3) and 2 enzymes capable of adding a β-glycosidic galactose residue to 5-hydroxylysine (GLT25D1 and GLT25D2, also called COLGALT1 and COLGALT2). In addition, PLOD3 can transfer an

Abbreviations: ER, endoplasmic reticulum; P4HA, prolyl 4-hydroxylase alpha subunit; PLOD, procollagen-lysine, 2-oxoglutarate 5-dioxygenase; LH, lysyl hydroxylase; GLT25D, Glycosyltransferase 25 domain containing; COLGALT, Collagen beta(1-O)galactosyltransferase; P4HB/PDI, prolyl 4-hydroxylase beta subunit or protein disulfide isomerase; Gulo, Gulonolactone (L-) oxidase; DMOG, dimethyloxalylglycine; HSP47, heat shock protein 47; EDA, extra domain A; MMP, matrix metalloproteinase

* Corresponding author at: UCSD, Mail Code 0663, 9500 Gilman Dr., La Jolla, CA 92093-0663, United States.
E-mail address: rcowling@ucsd.edu (R.T. Cowling).
[1] Present address: Division of Cardiology, Cardiovascular Center, Veterans Health Service (VHS) Medical Center, Seoul, Korea.
[2] Present address: The Johns Hopkins School of Medicine, 733 Rutland Avenue, Baltimore, MD 21205-2109.

http://dx.doi.org/10.1016/j.yjmcc.2017.09.005
Received 11 August 2017; Received in revised form 13 September 2017; Accepted 14 September 2017
Available online 18 September 2017
0022-2828/

additional glucose residue to this galactose via α-glycosidic linkage. These enzymes act until triple helices form, at which time triple-helical procollagens are bound by the chaperone, HSP47, and exit the ER (reviewed in [9]). Ascorbate is a general antioxidant, but it is also a necessary cofactor for maximal procollagen hydroxylase activity, specifically to reactivate the enzyme after an "uncoupled reaction" [10]. Other reductants appear able to substitute for ascorbate both in vitro and in vivo [11–17], but ascorbate appears to be the most efficacious.

Cardiac fibroblasts are routinely cultured in the absence of ascorbate, unless the secretion of correctly folded triple helices is to be studied. More than 30 years ago, Murad et al. noted that culturing human skin fibroblasts with newly added ascorbate resulted in a ~6-fold reduction in intracellular prolyl hydroxylase activity between 24 and 96 h after ascorbate addition, although the mechanisms and implications were not examined further [18]. Cardiac fibroblasts should be able to survive and deposit collagen within hostile environments, where nutrient levels, including those of ascorbate, may be limiting. We hypothesized and have shown, herein, that ascorbate deprivation has profound effects on the metabolism of procollagens within the cardiac fibroblasts, forcing them to adapt to this situation and altering their physiology. The implications of our findings for the study of cardiac fibroblasts and the understanding of the complex mechanisms of collagen synthesis in the heart are discussed.

2. Materials and methods

2.1. Preparation and culturing of adult cardiac fibroblasts

Adult male Sprague Dawley rats (200–250 g) were purchased from Harlan Laboratories. Adult male C57Bl/6 mice (8 weeks old) were purchased from the Jackson Laboratory. Animals were handled using procedures that were approved by the UCSD Institutional Animal Care and Use Committee (IACUC). Non-myocytes were harvested from the ventricles of the mice or the left ventricles of the rats by proteolytic digestion and selective plating, as described previously [19]. Second passage cells (mostly fibroblasts and myofibroblasts) were routinely used for experiments. Standard tissue culture media was high glucose DMEM (Life Technologies cat #11965-092) + 10% fetal bovine serum (FBS) + antibiotic/antimycotic without ascorbate, unless otherwise noted.

2.2. Collagenase-sensitive [^{3}H]-proline incorporation

Cells were radiolabeled with L-[2,3,4,5-^{3}H]-proline (PerkinElmer NEN, NET483) in DMEM + 10% FBS for 16 h. Media was collected and debris removed by centrifugation for 5 min at 1000 rpm and room temperature. Five hundred microliters of supernatant was diluted with an equal volume of (10 mM $CaCl_2$, 100 mM Tris/HCl, pH 7.6 + 1 mg/mL collagenase type 2 (Worthington Biochemical Corporation)) and incubated at 37 °C for 60 min. The mixture was precipitated on ice for 10 min with 20% trichloroacetic acid. The precipitate was pelleted by centrifugation at maximum speed for 5 min at room temperature. The pellet was washed twice with ice-cold 10% trichloroacetic acid, twice with ice-cold acetone, air-dried, and dissolved in 500 μL (0.1 M NaOH, 0.5% SDS). Dpm (collagenase-resistant radiolabel) was determined by liquid scintillation counting. Total acid-precipitable radiolabel was determined by repeating the trichloroacetic acid precipitation without collagenase digestion. Collagenase-sensitive radiolabel secreted into the media was calculated as the difference between total and collagenase-resistant dpm. To correct for variations in cell density, the cells on the plate were washed with PBS, lysed in RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1.0% (v/v) IGEPAL® CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, containing protease inhibitors) on ice, cleared by centrifugation, and the total protein quantified using the Bio-Rad Protein Assay (cat #500-0006). Collagenase-sensitive dpm was normalized to total μg protein.

2.3. Western blotting

Cells were lysed with RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1.0% (v/v) IGEPAL® CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, containing protease inhibitors) on ice, cleared by centrifugation and total protein quantified using the Bio-Rad Protein Assay (cat #500-0006). Equal amounts of protein were electrophoresed on discontinuous SDS-PAGE and electrophoretically transferred to PVDF membranes. For immunoblotting of proteins secreted into the media, serum-free media was used to avoid electrophoretic problems caused by serum proteins. Media was collected and cleared by low-speed centrifugation. Secreted proteins were precipitated on ice with 20% trichloroacetic acid, washed with ice-cold 10% trichloroacetic acid, and washed with ice-cold acetone. Pellets were allowed to air-dry and then dissolved in SDS-PAGE sample buffer containing 1% 2-mercaptoethanol. Equal volumes were electrophoresed on discontinuous SDS-PAGE and electrophoretically transferred to PVDF membranes. Primary antibodies were as follows: GAPDH (Ambion #AM4300 mouse monoclonal 6C5 or Cell Signaling #2118 rabbit monoclonal antibody 14C10), P4HA1 (Novus Biologicals goat polyclonal antibody #NB100-57852), P4HA2 (Proteintech rabbit polyclonal antibody #13759–1-AP), P4HA3 (Proteintech rabbit polyclonal antibody #23185-1-AP), PLOD2 (Proteintech rabbit polyclonal antibody #13453-1-AP), PLOD3 (Proteintech rabbit polyclonal antibody #11027-1-AP), GLT25D1 (Proteintech rabbit polyclonal antibody #16768-1-AP), HSP47 (Santa Cruz Biotechnology mouse monoclonal antibody #sc-13150), Collagen α1(I), (Novus Biologicals rabbit polyclonal antibody #NBP1-30054, directed at the C-terminal telopeptide region), Collagen α2(I), (Proteintech rabbit polyclonal antibody #14695-1-AP), Collagen α1(III), (Proteintech rabbit polyclonal antibody #13548-1-AP), P4HB/PDI (Santa Cruz Biotechnology mouse monoclonal antibody #sc-74,551), and extra domain A (EDA)-fibronectin (Santa Cruz Biotechnology mouse monoclonal antibody #sc-59826). Secondary antibodies were species-matched horseradish peroxidase or alkaline phosphatase conjugates. For peroxidase, signals were visualized using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) and either X-ray film (qualitative assessment) or the C-DiGit Blot Scanner (LI-COR Biosciences, quantitative assessment). For phosphatase, signals were visualized using NBT/BCIP (Roche #11697471001).

2.4. Production of replication-deficient adenoviral vectors

Full-length mouse P4HA1 cDNA was purchased from ThermoFisher Scientific (MGC clone #3484815), and full-length mouse P4HA2 cDNA was purchased from GE Dharmacon (MGC clone #3157481). To remove the problematic *Pac*I site within the 3′-untranslated region of P4HA1, only the ~1800 bp *Eco*RI-*Psi*I ORF fragment was subcloned into the EcoRI-*Eco*RV sites of the shuttle vector, pENTR-1A. The entire ~2200 bp *Sal*I-*Not*I fragment of P4HA2 was subcloned into the SalI-NotI sites of pENTR-1A. To produce empty vector (MT), the ccdB gene was removed from pENTR-1A by *Eco*RI digestion, followed by intramolecular ligation. All three shuttle vectors were recombined individually into the adenoviral plasmid, pAd/CMV/V5-DEST, using the enzyme Gateway® LR Clonase® II. The resulting plasmids were linearized by *Pac*I digestion, packaged into Adenovirus-5, and titered on 293A cells. All procedures were followed according to manufacturer's instructions (Thermo Fisher Scientific). Both P4HA1 and P4HA2 were driven by a strong CMV promoter in the final vectors. The MT vector was used as a transduction control in experiments.

2.5. Statistical analyses

Where indicated, data are shown as the means ± standard errors (SE) of at least $n = 3$. Comparisons between means were performed with paired Student's *t*-tests, and among means with one factor analysis