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Original Contribution

H ferritin silencing induces protein misfolding in K562 cells: A Raman analysis

Fabiana Zolea [a,1], Flavia Biamonte [a,1], Patrizio Candeloro [b], Maddalena Di Sanzo [a], Anna Cozzi [c], Anna Di Vito [a], Barbara Quaresima [a], Nadia Lobello [a], Francesca Trecroci [a], Enzo Di Fabrizio [b,d], Sonia Levi [c,e], Giovanni Cuda [a,2], Francesco Costanzo [a,2,*]

[a] Department of Experimental and Clinical Medicine, Magna Græcia University of Catanzaro, Salvatore Venuta Campus, Catanzaro, Italy, 88100
[b] BioNEM Laboratory, Department of Experimental and Clinical Medicine, Magna Græcia University of Catanzaro, Salvatore Venuta Campus, Catanzaro, Italy, 88100
[c] San Raffaele Scientific Institute, Division of Neuroscience, Milano, Italy, 20132
[d] Physical Science & Engineering Division, King Abdullah University of Science and Technology (KAUST), Thuwal, Kingdom of Saudi Arabia, 23955-6900
[e] University Vita-Salute San Raffaele, Milano, Italy, 20132

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ABSTRACT

The redox state of the cell is involved in the regulation of many physiological functions as well as in the pathogenesis of several diseases, and is strictly dependent on the amount of iron in its catalytically active state. Alterations of iron homeostasis determine increased steady-state concentrations of Reactive Oxygen Species (ROS) that cause lipid peroxidation, DNA damage and altered protein folding. Ferritin keeps the intracellular iron in a non-toxic and readily available form and consequently plays a central role in iron and redox homeostasis. The protein is composed by 24 subunits of the H- and L-type, coded by two different genes, with structural and functional differences. The aim of this study was to shed light on the role of the single H ferritin subunit (FHC) in keeping the native correct protein three-dimensional structure. To this, we performed Raman spectroscopy on protein extracts from K562 cells subjected to FHC silencing. The results show a significant increase in the percentage of disordered structures content at a level comparable to that induced by H_2O_2 treatment in control cells. ROS inhibitor and iron chelator were able to revert protein misfolding. This integrated approach, involving Raman spectroscopy and targeted-gene silencing, indicates that an imbalance of the heavy-to-light chain ratio in the ferritin composition is able to induce severe but still reversible modifications in protein folding and uncovers new potential pathogenetic mechanisms associated to intracellular iron perturbation.

1. Introduction

Iron is a fundamental cofactor for many biochemical activities in living cells, since it is involved, among others, in energy metabolism, DNA synthesis and oxygen transport. On the other hand, free iron is potentially toxic for the cells since it may catalyse, through Fenton chemistry, the generation of Reactive Oxygen Species (ROS) that damage the structure of DNA, lipids and proteins; all cells have therefore developed a coordinated system of iron-uptake and iron-storage molecules. Ferritin, a globular protein of 450 kDa, localized in eukaryotic cells in cytoplasm, nucleus and mitochondria, fulfils the task of preserving iron in a non-toxic and readily available form.

The cytoplasmic molecule is a heteropolymer of 24 subunits of heavy- and light-type arranged in a hollow spherical shell with a central cavity of about 80 Å of diameter that may store up to 4500 atoms of iron [1], while in the nucleus the shell is only composed by H-type subunits [2]. The mitochondrial ferritin (FtMt) is a homopolymer of a single subunit with a 75% sequence identity to the H ferritin, encoded by an intronless gene, located on chromosome 5q23 in human [3].

The ferritin light chain (L; FLC) is coded by a gene [4] located on chromosome 19, and the ferritin heavy chain (H; FHC) is coded by a gene [5] that maps on chromosome 11. FHC has an enzymatic

Abbreviations: 2'-7'-DCF, 2'-7'-dichlorodihydrofluorescein; AβP, β-amiloid protein; BCA, bicinchoninic acid; CM-H2CFDA, 5-(and-6)-carboxy-2', 7'dichlorofluorescein; DCDR, drop coating deposition Raman; EMSC, extended multiplicative signal correction; FHC, ferritin heavy chain; Fth, ferritin H gene; Ftl, ferritin L gene; FtMt, ferritin mitochondrial; H-bonds, hydrogen bonds; HBSS, Hanks balanced saline solution; H-π, π hydrogen; PD, Parkinson's disease; PFA, paraformaldehyde; PrPc, prion protein; PrPsc, PrP-scrapie; shFHC, short hairpin FHC; shRNA, short hairpin RNA; SiCaF2, calcium floride

* Correspondence to: Full Professor of Biochemistry, Magna Græcia University, Catanzaro, Italy, 88100. Tel.: +3909613694700/01. Fax: +3909613694703.
E-mail address: fsc@unicz.it (F. Costanzo).

[1] These authors contributed equally to this work.
[2] These authors also contributed equally to this work.

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activity that specifically oxidizes ferrous iron Fe^{2+} to ferric iron Fe^{3+}, while FLC is mainly associated with iron nucleation and stabilization of assembled ferritin proteins [1].

An intriguing aspect of the cytoplasmic ferritin is represented by the fact that the ratio of the H to L subunits is not fixed, therefore resulting in a wide combination of H-L chains. It has been largely demonstrated that the structural differences in H/L ratio may depend on: (i) the type of tissue; (ii) the differentiation/proliferation state of the cell; (iii) the cell response to environmental signals; (iv) neoplastic transformation [6]. Until now, the molecular basis underlying the existence of such a large variety of isoforms has not been completely clarified.

As already reported, the two ferritin subunits play different roles in relation to iron storage and detoxification within the cell. As a consequence, an imbalance in their ratio has the potential to alter the intracellular iron content and availability: ferritin molecules in cells containing high levels of iron, such as liver and spleen, tend to be L-rich, and may have a long-term storage function, whereas H-rich ferritins are more active in rapid iron uptake and release and lead to a cell phenotype more responsive to acute environmental changes [7].

In the present work, we combined the gene silencing strategy with Raman spectroscopy, a technique developed to analyze vibrational, rotational and other low-frequency modes, to detect the effects of H ferritin knock-down, via ROS production, on the overall proteins structure in K562 cells. The analysis was achieved by mathematical decomposition of Amide I Raman band, which is well known to be sensitive to protein secondary structure. The data collected indicate that the H-ferritin silencing is accompanied by significant modifications of native protein folding, reversible when ROS are removed. The coupling of gene-specific modifications with a Raman analysis broadens the possibilities to study the biochemical pathways in which a given gene is involved.

2. Materials and methods

2.1. Cell culture, lentiviral transduction and protein extracts

K562 cells, a human cell line established from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises (ATCC number:CCL 243), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (Sigma Aldrich, St. Louis, MO) at 37 °C in an atmosphere of humidified air containing 5% CO_2. Lentiviral preparations and transductions were performed as previously described [8] using a shRNA as control or a shFHC that targets the 196–210 region of the FHC mRNA. All the experiments were performed using a puromycin-selected pool of clones (1 μg/ml) (Sigma Aldrich, St. Louis, MO). Protein extractions were peformed on K562 shRNA and shFHC treated or not with N-acetyl-cysteine 5 mM for 30 min or with deferoxamine (desferal) 200 μM for 1 or 5 h (Sigma Aldrich, St. Louis, MO). Briefly, for total protein extractions, K562 cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (20 mmol/L Tris, 150 mmol/L NaCl, 1% Igepal, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 0.15 units/mL aprotinin, and 10 μmol/L leupeptin) (Sigma Aldrich, St. Louis, MO) and after removal of the cell debris by centrifugation (12,000 × g, 30 min), the protein content was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

2.2. RNA extraction and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted with the TRizol RNA isolation system (Invitrogen, Carlsbad, CA). All the RNA samples were DNase-1 treated (Ambion, Austin, TX), and purity and integrity of the RNA was checked spectroscopically and by gel electrophoresis before use. One microgram of RNA from each sample was used for RT-PCR with a reverse transcriptase system kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using SYBR Green universal PCR master mix (Bio-Rad Laboratories, Hercules, CA) using FHC-specific primers: forward, 5'-cat caa ccg cca gat caa c-3'; reverse, 5'-gat ggc ttt cac ctg ctc at-3'. Each sample was normalized to its glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content. Relative gene expression for FHC mRNA was normalized to a calibrator that was chosen to be the basal condition (K562 shRNA). Results were calculated with the $\Delta\Delta$Ct method and expressed as n-fold differences in FHC gene expression relative to GAPDH and calibrator and were determined as follows:

$$n - \text{fold} = 2^{-\Delta\Delta Ct} (\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ calibrator})$$

where the parameter Ct (threshold cycle) is defined as the fractional cycle number at which the PCR reporter signal passes a fixed threshold. ΔCt values of the sample and calibrator are determined by subtracting the average Ct value of the transcript under investigation from the average Ct value of the GAPDH gene, for each sample.

2.3. Immunofluorescence

Cells were fixed with 4% paraformaldehyde (PFA) (Sigma Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) (Sigma Aldrich, St. Louis, MO) for 10 min at room temperature (RT) and permeabilized with 0,5% saponin (Sigma Aldrich, St. Louis, MO) in PBS for 30 min at room temperature. Samples were blocked for 30 min in blocking buffer (10% BSA in PBS) (Sigma Aldrich, St. Louis, MO). Primary antibody (ferritin heavy chain H-53, 1:100 Santa Cruz Biotechnology) was diluted in blocking buffer and incubated for 1 hr at 37 °C. To visualize binding, an appropriate secondary antibody (Alexa Fluor 488, 1:400, Invitrogen, Carlsbad, CA) diluted in blocking buffer was applied for 30 min at 37 °C. Nuclear Dapi (1:1000, Invitrogen, Carlsbad, CA) was added for 20 min after secondary antibody incubation, prior to washing and mounting (ProLong Gold antifade Reagent, Molecular Probes, Eugene, OR). Images were collected using a Leica TCS SP2 confocal microscopy system (63X objective).

2.4. ROS detection

ROS were determined by incubating K562 shRNA and shFHC cells with the redox-sensitive probe 2'-7'-DCF (CM-H_2CFDA; Molecular Probes, Eugene, OR). Briefly, 1×10^6 K562 cells were plated in 96-well plates and incubated with Hanks balanced saline solution (HBSS), 10 mM glucose and 20 μM DCF for 15 min at 37 °C. After two cycle washes, cells were maintained in HBSS supplemented with 10 mM glucose. Fluorescence was revealed using the Victor3 Multilabel Counter (Perkin Elmer, Turku, FI) at 485 nm and 535 nm for excitation and emission, respectively. Results were normalized on protein concentration evaluated by the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Waltham, MA).

2.5. Raman measurements

Raman spectra on proteins extractions from the different cell lines have been recorded by means of an InVia Raman microscope from Renishaw (Renishaw Apply Innovation, New Mills, UK), equipped with an 832 nm laser source and in backscattering configuration. All Raman measurements are recorded with a total laser power of about 10 mW at the sample level and an