Biochimica et Biophysica Acta 1850 (2015) 2177–2184

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Using an in vitro model to study oxidised protein accumulation in ageing fibroblasts

Nae Shiozawa-West, Rachael A. Dunlop, Kenneth J. Rodgers *

The Cell Biology Group, School of Life Sciences, University of Technology Sydney, Ultimo, NSW 2007, Australia

ARTICLE INFO

Article history:
Received 11 January 2015
Received in revised form 2 July 2015
Accepted 10 July 2015
Available online 15 July 2015

Keywords:
Oxidised proteins
Ageing
DOPA
Proteasome
Lysosomes

ABSTRACT

Background: The accumulation of oxidised proteins in ageing cells and tissues results from an increase in oxidant damage coupled with impaired degradation of the damaged proteins. Heat Shock Proteins (HSP) and other chaperones are required to recognise damaged proteins and transport them to the lysosomal and proteasomal degradation pathways. How these systems fail in ageing cells is not clear.
Methods: We monitor oxidised protein accumulation, the activity of the proteasome and lysosomal proteases, and HSP levels in MRC-5 fibroblasts throughout their mitotic lifespan. We then use a novel in vitro cell culture model to experimentally generate oxidised proteins in young and old MRC-5 fibroblasts and compare their rates of degradation and changes in the key pathways involved in oxidised protein removal.
Results: We show that the activity of the proteasome and some lysosomal enzymes decreases with ageing in MRC-5 cells as do levels of HSP70 but this is not associated with an accumulation of oxidised proteins which only occurs as cells closely approach post-mitotic senescence. Old cells are unable to degrade experimentally generated oxidised proteins as efficiently as young cells. Exposure to mild heat stress however increases the efficiency of oxidised protein degradation by young cells and increases levels of HSP70.
Conclusions: Our results highlight the importance of the HSP/chaperone system in oxidised protein metabolism, particularly in ageing cells.
General significance: These data might have implications for the development of therapies for pathologies associated with protein accumulation and suggest that the HSP/chaperone system would be an important target.

1. Introduction

The accumulation of oxidised proteins and a gradual decline in proteasome [1–4] and lysosome function [5–8] are features of cellular ageing. The accumulation of oxidised proteins results from an increase in oxidant damage coupled with the less efficient removal of the damaged proteins [9]. Removal by proteolysis is the primary defence against the accumulation of oxidised proteins [10]. Protein oxidation can result in protein unfolding which can expose previously buried hydrophobic regions and, if not rapidly recognised by heat shock proteins (HSPs), unfolded proteins can form aggregates through hydrophobic interactions [10]. Once this process is initiated, aggregates can grow and be stabilised by covalent cross-links, eventually rendering them protease-resistant. Failure of HSPs to rapidly recognise, bind to, and transport oxidised proteins to the degradation machinery can potentially result in protein aggregation and accumulation even when the proteolytic machinery is fully active. Since oxidised and aggregated proteins can directly inhibit the proteasome [11,12] and lysosomal cathepsins [13] it is difficult to determine to what extent loss of proteolytic activity in ageing is due to inhibition from accumulating oxidised and aggregated proteins. Impairment in the activity of the degradation pathways might not, by itself therefore, account for the extent of oxidised protein accumulation reported in ageing. HSPs and other chaperones are required to recognise and target damaged proteins to both the lysosomal [14] and proteasomal pathways [15,16], failure of this quality control system to rapidly bind damaged proteins increases the likelihood of protein aggregation, cross-linking and resistance to proteolysis. Attenuated induction of HSP expression has been associated with fibroblast ageing in vitro and with the age of primary cells obtained from donors [17,18].

Naturally ageing human diploid fibroblast cells eventually reach their post-mitotic, Hayflick limit of replicative capacity and enter irreversible growth arrest or senescence. In the present studies we monitor oxidised protein levels in ageing MRC-5 cells and correlate this with changes in proteolytic activity and levels of HSPs. To generate 'oxidised proteins' in cells we use a model we have developed that allows cells to biosynthetically incorporate L-3,4-dihydroxyphenylalanine (DOPA) into proteins. DOPA is the primary oxidation product of hydroxyl radical attack on tyrosine residues and is present in proteins in tissues from

Abbreviations: DOPA, L-3,4-dihydroxyphenylalanine; HSPs, heat shock proteins; HPLC, high performance liquid chromatography; PD, population doubling.

* Corresponding author at: School of Life Sciences, University of Technology Sydney, Faculty of Science, Building 4, Level 7, Thomas Street, Ultimo, NSW 2007, Australia.
E-mail address: Kenneth.rodgers@uts.edu.au (K.J. Rodgers).

http://dx.doi.org/10.1016/j.bbagen.2015.07.002
0304-4165/

age-related diseases such as atherosclerosis [19] and cataractogenesis [20]. We have previously demonstrated that DOPA can be mistakenly incorporated into cell proteins during protein synthesis and replaces the protein amino acid L-tyrosine [21,22]. Incorporation of DOPA into proteins can lead to protein misfolding and exposure of previously buried hydrophobic regions resulting in loss of solubility [23]. DOPA is also a potent cross-linker and can form cross-links with histidine residues [24] and cysteine residues [25]. This provides a useful in vitro model to study protein misfolding and aggregation. We use this model system to examine the effects of DOPA-containing proteins, on the protein degradation pathways in young and old fibroblasts. We investigate how HSP levels change with ageing and demonstrate that mild heat stress can reduce the accumulation of oxidised proteins in this model.

2. Material and methods

2.1. Reagents

EMEM (Eagle's Minimal Essential Medium) deficient in tyrosine, phenylalanine and Phenol Red was from JRH Biosciences. L-[3-^{14}C-alanine] dopa and L-[U-^{14}C] leucine were from Amersham Biosciences (GE Healthcare). *N*-succinyl-Leu-Leu-Val-Tyr-AMC (where Suc is succinyl and AMC is 7-amino-4-methylcoumarin) and Boc-Leu-Ser-Thr-Arg-AMC (where Boc is t-butoxycarbonyl) were purchased from Sigma Chemical Co. Z-Arg-Arg-AMC (where Z is benzyloxycarbonyl), Z-Phe-Arg-AMC and Ac-Nle-Pro-Nle-Asp-AMC (where Ac is acetyl and Nle is norleucine) were from Bachem AG. Anti-Hsp70 (cat# 386013) and anti-Hsp27 rabbit polyclonal (cat# 386035) were purchased from Calbiochem, Darmstadt, Germany. Anti-Hsp90 rabbit polyclonal (cat# ab53110) and HRP-conjugated anti-rabbit IgG H&L goat polyclonal secondary antibody (cat# ab6721) was purchased from Abcam Inc, Cambridge, UK.

All aqueous solutions and buffers were prepared using water filtered through a four-stage Milli-Q system (Millipore). All other chemicals, solvents and chromatographic materials were of analytical reagent or cell-culture grade.

2.2. Cell culture

MRC-5 cells, a human lung fibroblast cell line (ATCC® number, CCL-171) were cultured in EMEM supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin (concentrated premix; Cambrex Bio Science; MD, USA) and 2 mM L-glutamine. Cells were maintained at 37 °C with 5% CO_2.

For studies involving the synthesis of DOPA-containing proteins, cells were cultured in tyrosine-deficient EMEM supplemented with a range of concentrations (50–750 μM) of L-DOPA over 4–24 h. As negative controls, cells were incubated in tyrosine-deficient EMEM. Medium also contained 10% heat-inactivated FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin (premixed) and 2 mM L-glutamate. After incubations, medium was removed and the MRC-5 cells harvested by trypsin-EDTA. Cell pellets were then collected by centrifugation at 2500 rpm for 5 min, washed twice with phosphate buffered saline (PBS) and stored at −80 C until required. The extent of DOPA incorporation into cellular proteins is expressed as a ratio mole per mole of tyrosine.

The cells subjected to mild heat stress were placed at 45 °C for 15 min in atmospheric CO_2 prior to treatment with DOPA.

2.3. HPLC analysis

Cell were washed and lysed by three freeze thaw cycles. Proteins were isolated by precipitation in TCA (5%), delipidated and washed by resuspending twice in 5% TCA containing 0.02% sodium deoxycholate and 0.15 mg/mL sodium borohydride (reducing agent) then washed twice with ice-cold acetone. The protein pellets were hydrolysed under anaerobic conditions (HCl and β-mercaptoacetic acid) using a standard gas-phase acid-catalysed method [26]. The level of DOPA in protein was determined by reverse-phase HPLC, using a LC-10A system from Shimadzu Co. (Kyoto, Japan) equipped with a CTO-10ASvp column oven (Millipore Co.) set at 30 °C and methods described previously [19,21]. System operation was automated by Class LC-10 software. Chomatography was performed on a Zorbax ODS column with an attached Pelliguard guard column (LC-18). The elution was performed using a binary gradient of Buffer A (100 mM sodium perchlorate and 10 mM sodium phosphate buffer, pH 2.5) and Buffer B (80% (v/v) methanol) at a flow rate of 1 mL/min as follows: isocratic elution with 4% Buffer B for 25 min, 10% Buffer B for 5 min, increase to 40% Buffer B and maintained 40% Buffer B for 20 min, re-equilibration at 1% Buffer B for the final 10 min. UV λ280 nm (Shimadzu Co.), fluorescence (λ_{ex}280nm and λ_{em} 320 nm, Hitachi F-1080, Tokyo, Japan) and electrochemical detector (ECD; Antec Leyden BV, Zoeterwoude, Netherlands) with the electrode potential set at 1.2 V measurements of the eluent were monitored in series. The amount of oxidised derivative incorporated into proteins was quantified from standards used, calculated as a ratio of mol oxidised derivative to mol of parent amino acid tyrosine.

2.4. Activity studies

All assays were carried out in 96-well plates in triplicates and no protein control was included. Change in fluorescent or colourimetric unit/min/protein (g) was calculated and expressed as a percentage (%) of control values. The protein concentration was determined using the Bradford-based total protein assay.

2.5. Measurement of proteasome activity

Proteasome chymotryptic, peptidylglutamyl-peptide-hydrolysing (PGPH) and tryptic activities were measured by the initial linear rates of cleavage of the fluorescent reporter group (7-amino-4-methylcoumarin/AMC) from peptide substrates *N*-Succinyl-Leu-Leu-Val-Tyr-AMC (Sigma-Aldrich Co., MO, USA), Acetyl-Nle-Pro-N [27] le-Asp-AMC (where Nle is norleucine; Bachem Holding AG, Bubendorf, Switzerland), Boc-Leu-Ser-Thr-Arg-AMC (where Boc is t-butoxycarbonyl; Sigma-Aldrich Co.) respectively (previously described in [22,27]). Pelleted cells were resuspended in homogenising buffer containing 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.025% digitonin, 0.5 mM EDTA and 50 mM Tris–HCl, pH 7.5 at 4 °C. This homogenate was incubated on ice for 5 min to allow digitonin-mediated permeabilisation of the cell membrane followed by centrifugation at 20,000 ×*g* for 15 min at 4 °C. Samples were incubated for 30 min at room temperature in the presence or absence of the proteasome inhibitor epoxomicin (20 μM). Proteasome activity was measured in reaction buffer (0.05 mg/mL BSA, 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT and 50 mM Tris–HCl, pH7.5) with 100 μM chymotryptic/PGPH or 600 μM for the tryptic substrates by measuring the change in fluorescence (λ_{ex}360 nm and λ_{em}469 nm) for 30 min.

2.6. Measurement of cathepsin L, B and S activities

Cathepsin L, B and S activity were measured by the initial linear increase in fluorescence following the cleavage of AMC from peptide substrates Z-Phe-Arg-AMC (where Z is benzyloxycarbonyl), Z-Arg-Arg-AMC and Z-Val-Val-Arg-AMC (all from Bachem Holding AG) respectively [22]. For cathepsins L and B, 1/21 of final volume of cell lysate and reaction buffer (2.5 mM DTT, 5 mM EDTA, 1 μM pepstatin A, 5 mM benzamidine, 0.1 M phosphate buffer pH 6 (cathepsin B) or pH 5.5 (cathepsin L) containing 0.005% Brij 35) were incubated for 5 min at room temperature on a shaker. 200 μM cathepsin B or L substrate was added to a final volume of 210 μL per assay and the fluorescence was