# USING AN IN VITRO MODEL TO STUDY OXIDISED PROTEIN ACCUMULATION IN AGEING FIBROBLASTS

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Running title: DOPA in ageing cells

## Highlights

There is a decline in proteasome activity and levels of HSP70 with MRC-5 cell aging

Cells utilise L-DOPA for L-tyrosine to generate ‘oxidised’ aggregate-prone proteins

Older MRC-5 cells degrade DOPA-containing proteins less efficiently than young cells

HSP70 levels in older MRC-5 cells increases in response to DOPA and heat stress.

Mild heat stress increases oxidised protein turnover in older MRC-5 cells

**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 aging 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.

Key words: Oxidised proteins, aging, DOPA, proteasome, lysosomes

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

## 1. Introduction

The accumulation of oxidised proteins and a gradual decline in proteasome [1-4] and lysosome function [5-8] are features of cellular aging. 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 aging. 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 aging *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 aging 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 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 hours. 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 2,500 rpm for 5 min, washed twice with phosphate buffered saline (PBS) and stored at -80 ℃ 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 minutes 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 ($\lambda_{ex}$280nm and $\lambda_{em}$ 320nm, 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 x 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 ($\lambda_{ex}$360 nm and $\lambda_{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 measured ($\lambda_{ex}$360 nm and $\lambda_{em}$469 nm) for 30 min. For cathepsin S, different reaction buffer was used (5 mM benzamidine, 5 mM EDTA, 1 μM pepstatin A, 0.01% Triton X-100, 1 mM PMSF, 5 mM DTT and 0.2 M phosphate buffer, pH 7.5) and was incubated for 60 min at 40°C and the fluorescence was measured for 50 min.

*2.7 Measurement of arylsulphatase activity*

Arylsulphatase activity was measured by hydrolysis of the ester sulphate bond of the fluorogenic substrate, *p*-nitrocatecholsulphate [28]. 19x volume of reaction buffer (0.1% Triton X-100, 7.5 mM *p*-nitrocatecholsulphate and 0.1 M sodium acetate buffer, pH 5.0) was added to cell lysate and incubated at 37°C for 30 min. The reaction was terminated with the addition of an equal amount of stop buffer (4.76 mM HCl containing 0.19% (w/v)

hydroquinone freshly mixed 1:1 with 2.38 M NaOH containing 4.76% $Na_2SO_3$). Absorbance was measured at λ540 nm.

*2.8 Measurement of acid phosphatase activity*

Acid phosphatase activity was measured in cell lysates by hydrolysis of the phosphate group from the fluorogenic substrate, *p*-nitrophenyl phosphate. 4x volume of reaction buffer (8 mM *p*-nitrophenyl phosphate, 90 mM sodium acetate-acetic acid, pH 5.0) was added to each well then the plate incubated at 37°C for 30 min. The reaction was terminated by the addition of NaOH stop buffer to a final concentration of 176 mM NaOH. Absorbance was measured at λ410 nm.

*2.9 Measurement of lysosomal acid lipase activity*

Lysosomal acid lipase activity was estimated in the cell lysates by hydrolysis of the fluorogenic substrate, 4-methylumbelliferyl-oleate [29]. Initially, 4-methylumbelliferyl-oleate is resuspended in hexane (100 mg/mL) and then diluted 1 in 100 in 4% Triton X-100. 2x the volume of 4-methylumbelliferyl-oleate stock and 5x volume of assay buffer (0.2 M sodium acetate and 0.001% Tween 20, pH 5.5) were added to samples and incubated at 37°C for 30 min; the reaction was stopped by adding a half volume of 0.75 M Tris, pH 8.0. Fluorescence measurements were measured with $\lambda_{ex}$360 nm and $\lambda_{em}$469 nm.

*2.10 Degradation studies*

The rate of degradation of bulk proteins containing L-[U-$^{14}$C]leucine ($^{14}$C-leucine) or

L-3,4-dihydrophenyl[3-$^{14}$C]alanine ($^{14}$C-DOPA), synthesised in the presence of DOPA was measured. The specific activities of $^{14}$C-leucine and $^{14}$C-DOPA were 51.0 mCi/mmol and 306 mCi/mmol respectively, and the radioactive concentrations were 51.0 μCi/mmol and 250 μCi/mmol, respectively. Both radioactive labels were purchased from GE Healthcare. The cell culture method used to generate DOPA-containing proteins was as described previously [21].

Cells maintained in tyrosine- and leucine-free EMEM were incubated in the presence of 0.25 μM $^{14}$C-leucine and varying concentrations of DOPA (0, 250, 500, 750 μM) for 24 h. After 24 h of incubation, the medium was replaced with 'chase' medium; EMEM saturated with 10 μM leucine and 10 μM tyrosine, and cells were maintained in culture for a further 8 h to allow degradation of the labelled proteins. In experiments using $^{14}$C-DOPA, tyrosine-free EMEM contained 5 μM $^{14}$C-DOPA (with the balance of unlabelled DOPA) and EMEM saturated with 10 μM tyrosine was used as the 'chase' medium. Cells were harvested and the radioactivity in the culture medium and cell lysate measured by liquid scintillation counting. Bulk ($^{14}$C-leucine-labelled) and $^{14}$C-DOPA-containing protein degradation was measured from the release of free radioactivity into the culture medium. Degradation was expressed as a percentage of the free radioactivity counts in medium to the total number of counts in the system (ie the total pool of labelled proteins at the start of the degradation period).

*2.11 Fluorescence microscopy*

Cellular autofluorescence was imaged using an Olympus IX71 Inverted Research Fluorescence Microscope (Olympus Co., Tokyo, Japan) equipped with an NIBA filter unit ($\lambda_{ex}$470–490 nm, $\lambda_{em}$515–550 nm). Cells were exposed for 0.22 s. and images captured using the DP Controller software version 3.1.1.267 (Olympus Co.).

*2.12 Western blotting*

The SDS-polyacrylamide gel electrophoresis method described here was modified from the manufacturer's instructions for NuPAGE® Novex Bis-Tris gels (Invitrogen Co.). Cell pellets were lysed using the a Mammalian Cell Lysis kit (Sigma-Aldrich). Cell lysates were mixed with 4x NuPAGE® LDS sample buffer and 10x NuPAGE® reducing agent, denatured at 100°C for 5 min and immediately cooled on ice for 2 min. Samples containing up to 20 µg of protein were loaded per lane alongside 10 µL SeeBlue Plus 2 Pre-Stained molecular weight markers (4–250 kDa) onto a 10-well NuPAGE® Bis-Tris 4–12% pre-cast gel, 1.5 mm thick. The proteins were separated under denaturing conditions in NuPAGE® MES SDS Running Buffer, pH 6.4 with an addition of 500 µL NuPAGE® antioxidant to maintain reducing conditions. The electrophoresis was run at 100 V for 5 min, 150 V for 5 min followed by 200 V for 30 min for MES using the XCell SureLock$^{TM}$ Mini-Cell system (Invtirogen Co.). After SDS-PAGE separation, proteins were transferred onto nitrocellulose membranes, using the iBlot$^{TM}$ dry blotting system (Invitrogen Co.) and iBlot$^{TM}$ transfer stacks over 7 min using built-in 'P2' transfer program. Nitrocellulose membranes were blocked by incubating in 5% skim milk in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) overnight at 4°C and incubated with the primary antibody diluted in 0.1% TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) for 1 h at room temperature: rabbit polyclonal anti-HSP27 (Calbiochem #386013) was used at 1:10,000, rabbit polyclonal anti-HSP70 (Calbiochem #386035) was used as 1:50,000 and rabbit polyclonal anti-HSP90 (Abcam Inc. #ab53110) was used as 1:1,000.

Membranes were washed and incubated in secondary antibody conjugated to HRP diluted in 0.1% TBS-T for 1 h at room temperature on a rotating platform. HRP-conjugated anti-rabbit IgG H&L goat polyclonal secondary antibody from Abcam Inc. (cat. no. #ab6721)

at 1:10,000 and the membrane was incubated in HRP-ECL Western Blotting detecting solution as per manufacture's instructions (GE Healthcare) at room temperature for a maximum of 5 min. The detection reagent was removed and the membrane was exposed under chemiluminescence on the ChemiDoc XRS system (Bio-Rad Laboratories, Inc.). The relative expression of target protein was analysed using the densitometry function of QuantityOne$^{TM}$ software version 4.6.

*2.13 Statistical Analysis*

All results presented here as mean plus or minus standard error of the mean ($\bar{x}$±SEM). Statistical analyses were evaluated using GraphPad software (CA, USA) Prism 4 version 4.03 either using a one-way or a two-way ANOVA with Bonferroni multiple comparison post-tests to compare replicate means between different treatments across the samples. Differences were considered significant at $p < 0.05$.

**3. Results**

*3.1 DOPA-containing proteins accumulate with in vitro cellular aging.*

MRC-5 fibroblasts are capable of 42 to 46 population doublings before the onset of post-mitotic senescence. MRC-5 cells were initiated in culture at population doubling (PD) 10 and maintained until senescence was reached (approximately 6 months in culture). Cells were sampled at various PD times (203 separate lysates) and protein-bound DOPA quantitated as a measure of oxidative damage to proteins. DOPA was released from the protein chain by acid

hydrolysis [19] and levels quantitated using HPLC. DOPA levels (μmol DOPA per mol tyrosine) remained constant until cells entered the final phases of their mitotic life and a steep increase in DOPA levels in proteins was evident (Figure 1A: PD38: $P < 0.001$). This linear increase continued until cells entered post-mitotic senescence and did not undergo further division (PD42; $P < 0.001$, Figure 1A).

Post-mitotic cells were then maintained in culture for 3 months after becoming senescent. When viewed with microscopy, intense perinuclear fluorescence was present, consistent with the presence of protein aggregates (Figure 1B). This was not seen in young (PD16) or aging (PD36) cells (data not shown).

*3.2 Changes in HSP levels and proteasomal and lysosomal activity with aging.*

There was an overall attenuation of proteasomal catalytic activities in aging cells (PD36) when compared to young cells (PD16). The tryptic activity was the most significantly affected, falling to 43% (P = 0.0360), followed by the PGPH activity which was 66% (P = 0.0022) of that in young cells (Table 1). There was a trend towards reduced activity at the chymotryptic site (10% reduction in aging cells) but this did not reach significance (Table 1). Lysosomal protease and non-protease activities were also attenuated, reflecting a general decline in lysosomal function (to ~70%) in aging cells (Table 1).

Heat shock proteins have also been reported to decline with age, and we used western blotting to quantitate the levels of HSP27, HSP70 and HSP90 in cell lysates. Levels of HSP27 and HSP90 protein did not change with aging in MRC-5 fibroblasts (Figure 2B and C), however HSP70 protein levels were significantly (P < 0.001) decreased (Figure 2A).

*3.3 Generating DOPA-containing proteins in cells: a model for oxidized protein accumulation*

We experimentally generated DOPA-containing proteins in young (PD16) and aging (PD36) cells using a novel model developed in our laboratory [16,25]. In tyrosine-free medium the young rapidly dividing cells readily incorporated DOPA into newly synthesised proteins (Figure 3A) and even at 50 μM DOPA around 23% of tyrosine residues had been replaced with DOPA (Figure 3A). In older cells the rate of cell division and protein synthesis are much slower and the level of DOPA incorporation increased with increasing supply of DOPA in the medium (Figure 3A) and at 750μM DOPA around 9% of tyrosine residues had been replaced with DOPA (Figure 3A).

*3.4 Biosynthetically generated DOPA-containing proteins did not decrease cell viability*

To ensure the presence of DOPA-containing proteins did not adversely affect cell viability, we conducted LDH assays on young and aging cells. There was no increase in LDH release after 24 hours by young or aging cells when incubated with 750 μM DOPA or below (Figure 3B).

*3.5 Turnover of bulk proteins and DOPA-containing proteins in young and ageing cells*

To determine the effects of aging on the turnover of normal and DOPA-containing proteins, young (PD16) and aging (PD36) cells were incubated with increasing concentrations of DOPA, and the degradation of bulk cell proteins ($^{14}$C-leucine-labeled) and 'oxidized/damaged' proteins ($^{14}$C-DOPA-labeled proteins) measured. After a 24 hr protein labeling period, cells were subjected to a "chase period" consisting of an 8 hour incubation in medium containing tyrosine and lysine (25 mM) to allow the degradation of labelled proteins. Release of the radiolabeled amino acid was quantitated as described previously [25].

The degradation of bulk proteins (labeled with $^{14}$C-leucine) by aging cells (PD36) was significantly impaired at the highest concentration of DOPA (750 μM), when compared to young cells (PD16) (Figure 4A). This suggests that the aging cells were unable to maintain basal protein degradation rates when DOPA-containing proteins were present at higher levels. We then selectively examined the turnover of oxidised proteins, (labeled with $^{14}$C-DOPA) over the 8 hour chase period. The rate of removal of DOPA-containing proteins was significantly faster (~4 times) than bulk proteins (Figure 4B). Indicating that the proteolytic machinery of the cell specifically recognised DOPA-containing proteins as substrates for degradation. The rate of turnover of DOPA-containing proteins in aging cells however declined with increasing concentrations of DOPA to a greater extent than in young cells which were only able to maintain the higher level of turnover at concentrations up to 500 μM DOPA (Figure 4B).

*3.6 DOPA-containing proteins restored HSP70 levels in ageing cells*

In ageing cells levels of HSP70 protein had significantly declined by PD30 (Figure 2A). Treatment of PD36 cells with DOPA (500μg for 24 hours) had no effect on HSP levels in

young cells but selectively increased HSP70 levels in old cells effectively restored it to the level present in young cells (Figure 5).

*3.7 Mild heat stress improves the rate of turnover of DOPA-containing proteins*

We exposed old cells to mild heat stress by incubating them at 45°C for 15 min immediately prior to incubation with DOPA (0 – 24 hrs). We then compared the turnover of DOPA-containing proteins in aging cells that had been subjected to mild heat stress to control cells (without mild heat stress) and found there was a significant increase in the rate of degradation of DOPA-containing proteins at 750 μM in the heat stressed cells (Figure 6B). There was a marked increase in the expression of HSP70 by western blot after exposure to mild heat stress (Figure 6A) demonstrating that this was one factor that contributed to their increased ability to degrade the experimentally generated oxidised proteins.

## 4. Discussion

One of the most commonly observed changes in aging is the accumulation of damaged and aggregated material (lipofuscin/age-pigments) in cells, a large proportion of which is oxidised protein. The primary pathway for removal of oxidised proteins is degradation to their constituent amino acids, but this process appears to be impaired with aging and in some age-related diseases [30]. In the present studies we used an *in vitro* model system to examine the factors contributing to the decreased ability of ageing MRC-5 fibroblasts to degrade experimentally generated oxidised and aggregate-prone proteins.

Using protein-bound DOPA as a marker of oxidative damage to proteins, we monitored oxidised protein accumulation throughout the lifespan of MRC-5 cells. There was no increase in DOPA-containing proteins throughout most of the ageing process but a rapid increase in DOPA was evident in proteins when cells reached PD36 and the rate of cell division was greatly reduced. This is consistent with the idea that the distribution of oxidised proteins amongst daughter cells is an important mechanism for reducing oxidised protein accumulation in dividing cells. DOPA levels increased further as the cells entered post-mitotic senescence (PD42) (Figure 1A). The accumulation of DOPA-containing proteins corresponded to a decline in the activity of the proteasome and some lysosomal enzymes (Table 1) and a decline in levels of HSP70. Even although a significant decline in HSP70 protein levels were observed at PD32, levels of HSP27 and HSP90 were maintained even after senescence was reached suggesting that certain HSPs selectively decline with cellular aging.

We then generated DOPA-containing proteins in cells as a model of oxidised proteins. DOPA

is known to be the primary oxidation product of tyrosine residues in proteins and is present in proteins in ageing [20] and disease [19]. This approach has advantages over conventional methods of generating oxidised proteins since the use of strong oxidants such as paraquat [26] or hydrogen peroxide [27] can cause extensive damage to other biological molecules such as lipids and DNA [1,17]. Using the incorporation approach we can generate a precise pool of proteins containing oxidised amino acids without damage to other molecules and can thereby preserve cell viability. To do this we supplied DOPA to the cells in tyrosine-free medium so that DOPA could more effectively compete with tyrosine (present in the cell and in the foetal calf serum) for charging by tyrosyl tRNA synthetase and insertion into the polypeptide chain of proteins [16,25]. The presence of DOPA and DOPA-containing proteins had no effect on cell viability at the levels we used (Figure 3B). Incorporation of DOPA into proteins results in the generation of non-native/oxidised proteins in cells and thus might equate to a period of acute oxidative stress or an inflammatory insult *in vivo*. When DOPA-containing proteins were present in cells, both young and aging cells degraded these proteins much more rapidly than native ($^{14}$C leucine labeled) proteins (Figure 4A & B) proving evidence that the cells recognise at least some of the DOPA-containing proteins as damaged and selectively degraded them. We have previously demonstrated that degradation of DOPA-containing proteins by cells is biphasic, and at higher levels of DOPA incorporation, DOPA-containing proteins resist degradation [16] and aggregate [6]. In the present studies, at higher DOPA concentrations, there was a significant decline in the rate of degradation of DOPA-containing proteins in aging cells relative to young cells (Figure 4B). The ability of aging cells to degrade aggregate-prone, DOPA-containing proteins was clearly impaired. The two major pathways for the degradation of oxidized proteins are the proteasome and lysosomes. We showed that there was a reduction in the activity of the proteasome in aging cells compared to young cells where the trypsin-like activity was reduced to 43%, the PGPH to 66% and the

chymotrypsin to 89% (Table 1). The observation of an age-related reduction in proteolytic activity is supported by a large body of evidence [2,30] and this also applies in age-related pathologies [31–35]. A reduction in proteasome activity in aging cells also corresponded to a decline in the activity of the lysosomal enzymes; aryl sulphatase, acid phosphatase and acid lipase (Table 1). This may have been a result of an overall reduction in the population of lysosomes in the ageing cells.

There was a significant reduction in HSP70 protein levels in aging MRC-5 cells before there was any evidence of oxidised protein accumulation (Figure 2A). HSP70 is an inducible cytosolic heat shock protein that rapidly responds to thermal or oxidative stress, to sequester misfolded proteins by its binding domain, and thus preventing damaged proteins from forming aggregates. In this study we showed that HSP70 could be induced in aging cells both by exposure to DOPA or mild heat stress (Figures 5 and 6). Exposure to mild heat stress resulted in a small but significant increase in the rate of degradation of DOPA-containing proteins suggesting that HSP70 played a role in facilitating the degradation of these proteins (Figure 6B).

The accumulation of oxidised proteins in cells and tissues is a common feature of neurodegeneration and other disorders associated with aging. As was evident in the present studies, cell division and the distribution of oxidised material amongst daughter cells is an important mechanism that allows dividing cells to limit the accumulation of oxidised proteins [36,37]. The inability of senescent cells to divide could therefore account for the rapid accumulation of DOPA-containing proteins. This could occur in long-lived cells such as retinal pigment epithelial cells or neurons. Age pigments have also been shown to directly inhibit the function of the proteasomes and the lysosomes leading to a decline in cell function.

Whilst a great deal of research has focused on changes to the proteolytic machinery in aging

and neurodegeneration HSPs, which serve a critical upstream function, have received less attention. HSPs serve several roles in maintaining homeostasis including, protecting against aggregation, solubilizing of early stage protein aggregates, assisting in folding of nascent proteins or in refolding of damaged proteins. Importantly they also target damaged proteins to the degradation machinery and sequester non-degradable proteins to larger aggregates [12].

## Conclusions

By experimentally generating oxidised proteins in cells we showed that the basal turnover of cell proteins was maintained in ageing MRC-5 cells and they were able to efficiently degrade low levels of oxidised proteins. When higher levels of oxidised proteins were experimentally generated however their degradation in ageing cells was less efficient than in young cells. Impairment in proteasome activity and a decline in HSP function appeared to contribute to the decline in oxidised protein turnover. HSP70 levels were reduced in ageing cells but could be increased by mild heat stress resulting in an increased ability to degrade oxidise proteins. These studies describe a useful *in vitro* model to study the mechanisms of degradation of damaged or non-native proteins and highlight the importance of heat shock proteins in the removal of oxidized proteins and thus, the maintenance of homeostasis in aging and age-related pathologies.

## Acknowledgements

This work was supported by an NHMRC Australia project grant 189800.

**Legends to Figures**

**Table 1:** The peptidase activity of the proteasome (chymotrypsin-like, trypsin-like and PGPH activities), the lysosomal proteases (cathepsins B, L and S) and activity of lysosomal arylsulphatase, acid phosphatase and acid lipase in aging (PD36) MRC-5 cells and young (PD16) cells was compared. Activity was measured using fluorescently labelled substrates and set at 100% for young cells. Results represent triplicate sets of cultures and data was expressed as change in fluorescence per minute per mg of protein. * $p < 0.05$, ** $p < 0.01$, errors represent SEM.

**Figure 1:** A. Accumulation of protein-bound DOPA in ageing MRC-5 fibroblasts. DOPA in hydrolysed cell proteins was measured by HPLC and expressed as a ratio to tyrosine. B. At PD42 MRC-5 cells entered post-mitotic senescence. Cells were maintained in culture for more than 3 months after reaching post-mitotic senescence and native autofluorescence imaged using an inverted Olympus IX71 microscope with a NIBA filter set (ex 470–490nm, em 515–550nm) equipped with a DP71 digital camera with exposure of 0.22 seconds.

**Figure 2:** Densitometric analysis of HSP levels in MRC-5 cell lysates identified by western blotting. Cells were examined between PD13 and PD40 for levels of: A. HSP70, B. HSP90 and C. HSP27. Relative expression was quantified by densitometry (see insets). Statistically significant changes in the HSP protein levels relative to the young cells (PD16) were as indicated, ** $p < 0.01$, *** $p < 0.001$ and errors represent SEM.

**Figure 3:** A. Incorporation of DOPA into cell proteins from young (PD16) and aging (PD36) MRC-5 cells was measured. After incubation with DOPA (0 to 750μM) for 24 hours levels of DOPA in hydrolysed cell proteins was measured by HPLC (expressed as mMoles of DOPA per Mole tyrosine). B. The effect of DOPA on viability of young (PD16) and aging (PD36) cells was measured by quantifying LDH release from cells after incubation with DOPA for 24 hours. No decrease in cell viability was detected.

**Figure 4:** A. The degradation rate of $^{14}$C-leucine labelled (bulk) proteins was measured in young (PD16) and aging (PD36) MRC-5 cells over an 8 hour period. This was expressed as a percentage of the total pool of $^{14}$C-leucine labelled proteins generated in the preceding 24 hours. B. The degradation of $^{14}$C-DOPA labelled (oxidised) proteins was measured in young (PD16) and aging (PD36) MRC-5 cells over an 8 hour period. This was expressed as a percentage of the total pool of $^{14}$C-DOPA labelled proteins generated in the preceding 24 hours, * $p < 0.05$, **$p < 0.01$ and errors represent SEM.

**Figure 5:** Densitometric analysis of western blots of HSP70 protein levels in aging MRC-5 cells (PD36) exposed to DOPA (500μM) in tyrosine-depleted EMEM for 24 hours (Dark bars) compared to MRC-5 cells incubated in tyrosine-depleted EMEM for 24 hours without DOPA (Light bars). Relative expression of HSP27, HSP70 and HSP90 were measured by western blot and quantified by densitometry. Data are expressed relative to HSP protein levels in young cells (PD16). Results represent triplicate sets of cultures * $p < 0.05$ and error bars represent SEM.

**Figure 6:** A. Western blotting analysis of HSP70 protein levels in aging MRC-5 cells (PD36) exposed to mild heat stress (42˚C for 15 mins). B. MRC-5 cells (PD36) that had been pre-exposed to mild heat stress (labelled as mild stress) or control cells that were not exposed to mild heat stress (labelled control) were incubated with $^{14}$C-DOPA (5 μM) and increasing concentrations of non-labelled DOPA to give final concentrations 50 to 750 μM as indicated. The degradation rate of $^{14}$C-DOPA-labelled proteins was then measured over the following 8 hours in tyrosine saturated EMEM. Results represent triplicate sets of cultures ***$p < 0.001$ and errors represent SEM.

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Marked version: major changes are shown in blue text. Please note the manuscript has been re-structured and the results and discussion has been more clearly demarcated.

# USING AN IN VITRO MODEL TO STUDY OXIDISED PROTEIN ACCUMULATION IN AGEING FIBROBLASTS

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Running title: DOPA in ageing cells

**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 aging 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.

Key words: Oxidised proteins, aging, DOPA, proteasome, lysosomes

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

## 1. Introduction

The accumulation of oxidised proteins and a gradual decline in proteasome [1-4] and lysosome function [5-8] are features of cellular aging. 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 aging. 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 aging *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 aging 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 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 hours. 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 2,500 rpm for 5 min, washed twice with phosphate buffered saline (PBS) and stored at -80 ℃ 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 minutes 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 ($\lambda_{ex}$280nm and $\lambda_{em}$ 320nm, 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 x 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 ($\lambda_{ex}$360 nm and $\lambda_{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 measured ($\lambda_{ex}$360 nm and $\lambda_{em}$469 nm) for 30 min. For cathepsin S, different reaction buffer was used (5 mM benzamidine, 5 mM EDTA, 1 µM pepstatin A, 0.01% Triton X-100, 1 mM PMSF, 5 mM DTT and 0.2 M phosphate buffer, pH 7.5) and was incubated for 60 min at 40°C and the fluorescence was measured for 50 min.

*2.7 Measurement of arylsulphatase activity*

Arylsulphatase activity was measured by hydrolysis of the ester sulphate bond of the fluorogenic substrate, *p*-nitrocatecholsulphate [28]. 19x volume of reaction buffer (0.1% Triton X-100, 7.5 mM *p*-nitrocatecholsulphate and 0.1 M sodium acetate buffer, pH 5.0) was added to cell lysate and incubated at 37°C for 30 min. The reaction was terminated with the addition of an equal amount of stop buffer (4.76 mM HCl containing 0.19% (w/v)

hydroquinone freshly mixed 1:1 with 2.38 M NaOH containing 4.76% $Na_2SO_3$). Absorbance was measured at λ540 nm.

*2.8 Measurement of acid phosphatase activity*

Acid phosphatase activity was measured in cell lysates by hydrolysis of the phosphate group from the fluorogenic substrate, *p*-nitrophenyl phosphate. 4x volume of reaction buffer (8 mM *p*-nitrophenyl phosphate, 90 mM sodium acetate-acetic acid, pH 5.0) was added to each well then the plate incubated at 37°C for 30 min. The reaction was terminated by the addition of NaOH stop buffer to a final concentration of 176 mM NaOH. Absorbance was measured at λ410 nm.

*2.9 Measurement of lysosomal acid lipase activity*

Lysosomal acid lipase activity was estimated in the cell lysates by hydrolysis of the fluorogenic substrate, 4-methylumbelliferyl-oleate [29]. Initially, 4-methylumbelliferyl-oleate is resuspended in hexane (100 mg/mL) and then diluted 1 in 100 in 4% Triton X-100. 2x the volume of 4-methylumbelliferyl-oleate stock and 5x volume of assay buffer (0.2 M sodium acetate and 0.001% Tween 20, pH 5.5) were added to samples and incubated at 37°C for 30 min; the reaction was stopped by adding a half volume of 0.75 M Tris, pH 8.0. Fluorescence measurements were measured with $\lambda_{ex}$360 nm and $\lambda_{em}$469 nm.

*2.10 Degradation studies*

The rate of degradation of bulk proteins containing L-[U-$^{14}$C]leucine ($^{14}$C-leucine) or

L-3,4-dihydrophenyl[3-$^{14}$C]alanine ($^{14}$C-DOPA), synthesised in the presence of DOPA was measured. The specific activities of $^{14}$C-leucine and $^{14}$C-DOPA were 51.0 mCi/mmol and 306 mCi/mmol respectively, and the radioactive concentrations were 51.0 μCi/mmol and 250 μCi/mmol, respectively. Both radioactive labels were purchased from GE Healthcare. The cell culture method used to generate DOPA-containing proteins was as described previously [21].

Cells maintained in tyrosine- and leucine-free EMEM were incubated in the presence of 0.25 μM $^{14}$C-leucine and varying concentrations of DOPA (0, 250, 500, 750 μM) for 24 h. After 24 h of incubation, the medium was replaced with 'chase' medium; EMEM saturated with 10 μM leucine and 10 μM tyrosine, and cells were maintained in culture for a further 8 h to allow degradation of the labelled proteins. In experiments using $^{14}$C-DOPA, tyrosine-free EMEM contained 5 μM $^{14}$C-DOPA (with the balance of unlabelled DOPA) and EMEM saturated with 10 μM tyrosine was used as the 'chase' medium. Cells were harvested and the radioactivity in the culture medium and cell lysate measured by liquid scintillation counting. Bulk ($^{14}$C-leucine-labelled) and $^{14}$C-DOPA-containing protein degradation was measured from the release of free radioactivity into the culture medium. Degradation was expressed as a percentage of the free radioactivity counts in medium to the total number of counts in the system (ie the total pool of labelled proteins at the start of the degradation period).

*2.11 Fluorescence microscopy*

Cellular autofluorescence was imaged using an Olympus IX71 Inverted Research Fluorescence Microscope (Olympus Co., Tokyo, Japan) equipped with an NIBA filter unit ($\lambda_{ex}$470–490 nm, $\lambda_{em}$515–550 nm). Cells were exposed for 0.22 s. and images captured using the DP Controller software version 3.1.1.267 (Olympus Co.).

*2.12 Western blotting*

The SDS-polyacrylamide gel electrophoresis method described here was modified from the manufacturer's instructions for NuPAGE® Novex Bis-Tris gels (Invitrogen Co.). Cell pellets were lysed using the a Mammalian Cell Lysis kit (Sigma-Aldrich). Cell lysates were mixed with 4x NuPAGE® LDS sample buffer and 10x NuPAGE® reducing agent, denatured at 100°C for 5 min and immediately cooled on ice for 2 min. Samples containing up to 20 µg of protein were loaded per lane alongside 10 µL SeeBlue Plus 2 Pre-Stained molecular weight markers (4–250 kDa) onto a 10-well NuPAGE® Bis-Tris 4–12% pre-cast gel, 1.5 mm thick. The proteins were separated under denaturing conditions in NuPAGE® MES SDS Running Buffer, pH 6.4 with an addition of 500 µL NuPAGE® antioxidant to maintain reducing conditions. The electrophoresis was run at 100 V for 5 min, 150 V for 5 min followed by 200 V for 30 min for MES using the XCell SureLock™ Mini-Cell system (Invtirogen Co.). After SDS-PAGE separation, proteins were transferred onto nitrocellulose membranes, using the iBlot™ dry blotting system (Invitrogen Co.) and iBlot™ transfer stacks over 7 min using built-in 'P2' transfer program. Nitrocellulose membranes were blocked by incubating in 5% skim milk in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) overnight at 4°C and incubated with the primary antibody diluted in 0.1% TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) for 1 h at room temperature: rabbit polyclonal anti-HSP27 (Calbiochem #386013) was used at 1:10,000, rabbit polyclonal anti-HSP70 (Calbiochem #386035) was used as 1:50,000 and rabbit polyclonal anti-HSP90 (Abcam Inc. #ab53110) was used as 1:1,000.

Membranes were washed and incubated in secondary antibody conjugated to HRP diluted in 0.1% TBS-T for 1 h at room temperature on a rotating platform. HRP-conjugated anti-rabbit IgG H&L goat polyclonal secondary antibody from Abcam Inc. (cat. no. #ab6721)

at 1:10,000 and the membrane was incubated in HRP-ECL Western Blotting detecting solution as per manufacture's instructions (GE Healthcare) at room temperature for a maximum of 5 min. The detection reagent was removed and the membrane was exposed under chemiluminescence on the ChemiDoc XRS system (Bio-Rad Laboratories, Inc.). The relative expression of target protein was analysed using the densitometry function of QuantityOne™ software version 4.6.

*2.13 Statistical Analysis*

All results presented here as mean plus or minus standard error of the mean ($\bar{x}$±SEM). Statistical analyses were evaluated using GraphPad software (CA, USA) Prism 4 version 4.03 either using a one-way or a two-way ANOVA with Bonferroni multiple comparison post-tests to compare replicate means between different treatments across the samples. Differences were considered significant at $p < 0.05$.

**3. Results**

*3.1 DOPA-containing proteins accumulate with in vitro cellular aging.*

MRC-5 fibroblasts are capable of 42 to 46 population doublings before the onset of post-mitotic senescence. MRC-5 cells were initiated in culture at population doubling (PD) 10 and maintained until senescence was reached (approximately 6 months in culture). Cells were sampled at various PD times (203 separate lysates) and protein-bound DOPA quantitated as a measure of oxidative damage to proteins. DOPA was released from the protein chain by acid

hydrolysis [19] and levels quantitated using HPLC. DOPA levels (μmol DOPA per mol tyrosine) remained constant until cells entered the final phases of their mitotic life and a steep increase in DOPA levels in proteins was evident (Figure 1A: PD38: $P < 0.001$). This linear increase continued until cells entered post-mitotic senescence and did not undergo further division (PD42; $P < 0.001$, Figure 1A).

Post-mitotic cells were then maintained in culture for 3 months after becoming senescent. When viewed with microscopy, intense perinuclear fluorescence was present, consistent with the presence of protein aggregates (Figure 1B). This was not seen in young (PD16) or aging (PD36) cells (data not shown).

*3.2 Changes in HSP levels and proteasomal and lysosomal activity with aging.*

There was an overall attenuation of proteasomal catalytic activities in aging cells (PD36) when compared to young cells (PD16). The tryptic activity was the most significantly affected, falling to 43% (P = 0.0360), followed by the PGPH activity which was 66% (P = 0.0022) of that in young cells (Table 1). There was a trend towards reduced activity at the chymotryptic site (10% reduction in aging cells) but this did not reach significance (Table 1). Lysosomal protease and non-protease activities were also attenuated, reflecting a general decline in lysosomal function (to ~70%) in aging cells (Table 1).

Heat shock proteins have also been reported to decline with age, and we used western blotting to quantitate the levels of HSP27, HSP70 and HSP90 in cell lysates. Levels of HSP27 and HSP90 protein did not change with aging in MRC-5 fibroblasts (Figure 2B and C), however HSP70 protein levels were significantly (P < 0.001) decreased (Figure 2A).

*3.3 Generating DOPA-containing proteins in cells: a model for oxidized protein accumulation*

We experimentally generated DOPA-containing proteins in young (PD16) and aging (PD36) cells using a novel model developed in our laboratory [16,25]. In tyrosine-free medium the young rapidly dividing cells readily incorporated DOPA into newly synthesised proteins (Figure 3A) and even at 50 μM DOPA around 23% of tyrosine residues had been replaced with DOPA (Figure 3A). In older cells the rate of cell division and protein synthesis are much slower and the level of DOPA incorporation increased with increasing supply of DOPA in the medium (Figure 3A) and at 750μM DOPA around 9% of tyrosine residues had been replaced with DOPA (Figure 3A).

*3.4 Biosynthetically generated DOPA-containing proteins did not decrease cell viability*

To ensure the presence of DOPA-containing proteins did not adversely affect cell viability, we conducted LDH assays on young and aging cells. There was no increase in LDH release after 24 hours by young or aging cells when incubated with 750 μM DOPA or below (Figure 3B).

*3.5 Turnover of bulk proteins and DOPA-containing proteins in young and ageing cells*

To determine the effects of aging on the turnover of normal and DOPA-containing proteins, young (PD16) and aging (PD36) cells were incubated with increasing concentrations of DOPA, and the degradation of bulk cell proteins ($^{14}$C-leucine-labeled) and 'oxidized/damaged' proteins ($^{14}$C-DOPA-labeled proteins) measured. After a 24 hr protein labeling period, cells were subjected to a "chase period" consisting of an 8 hour incubation in medium containing tyrosine and lysine (25 mM) to allow the degradation of labelled proteins. Release of the radiolabeled amino acid was quantitated as described previously [25].

The degradation of bulk proteins (labeled with $^{14}$C-leucine) by aging cells (PD36) was significantly impaired at the highest concentration of DOPA (750 µM), when compared to young cells (PD16) (Figure 4A). This suggests that the aging cells were unable to maintain basal protein degradation rates when DOPA-containing proteins were present at higher levels. We then selectively examined the turnover of oxidised proteins, (labeled with $^{14}$C-DOPA) over the 8 hour chase period. The rate of removal of DOPA-containing proteins was significantly faster (~4 times) than bulk proteins (Figure 4B). Indicating that the proteolytic machinery of the cell specifically recognised DOPA-containing proteins as substrates for degradation. The rate of turnover of DOPA-containing proteins in aging cells however declined with increasing concentrations of DOPA to a greater extent than in young cells which were only able to maintain the higher level of turnover at concentrations up to 500 µM DOPA (Figure 4B).

*3.6 DOPA-containing proteins restored HSP70 levels in ageing cells*

In ageing cells levels of HSP70 protein had significantly declined by PD30 (Figure 2A). Treatment of PD36 cells with DOPA (500µg for 24 hours) had no effect on HSP levels in

young cells but selectively increased HSP70 levels in old cells effectively restored it to the level present in young cells (Figure 5).

*3.7 Mild heat stress improves the rate of turnover of DOPA-containing proteins*

We exposed old cells to mild heat stress by incubating them at 45°C for 15 min immediately prior to incubation with DOPA (0 – 24 hrs). We then compared the turnover of DOPA-containing proteins in aging cells that had been subjected to mild heat stress to control cells (without mild heat stress) and found there was a significant increase in the rate of degradation of DOPA-containing proteins at 750 μM in the heat stressed cells (Figure 6B). There was a marked increase in the expression of HSP70 by western blot after exposure to mild heat stress (Figure 6A) demonstrating that this was one factor that contributed to their increased ability to degrade the experimentally generated oxidised proteins.

## 4. Discussion

One of the most commonly observed changes in aging is the accumulation of damaged and aggregated material (lipofuscin/age-pigments) in cells, a large proportion of which is oxidised protein. The primary pathway for removal of oxidised proteins is degradation to their constituent amino acids, but this process appears to be impaired with aging and in some age-related diseases [30]. In the present studies we used an *in vitro* model system to examine the factors contributing to the decreased ability of ageing MRC-5 fibroblasts to degrade experimentally generated oxidised and aggregate-prone proteins.

Using protein-bound DOPA as a marker of oxidative damage to proteins, we monitored oxidised protein accumulation throughout the lifespan of MRC-5 cells. There was no increase in DOPA-containing proteins throughout most of the ageing process but a rapid increase in DOPA was evident in proteins when cells reached PD36 and the rate of cell division was greatly reduced. This is consistent with the idea that the distribution of oxidised proteins amongst daughter cells is an important mechanism for reducing oxidised protein accumulation in dividing cells. DOPA levels increased further as the cells entered post-mitotic senescence (PD42) (Figure 1A). The accumulation of DOPA-containing proteins corresponded to a decline in the activity of the proteasome and some lysosomal enzymes (Table 1) and a decline in levels of HSP70. Even although a significant decline in HSP70 protein levels were observed at PD32, levels of HSP27 and HSP90 were maintained even after senescence was reached suggesting that certain HSPs selectively decline with cellular aging.

We then generated DOPA-containing proteins in cells as a model of oxidised proteins. DOPA

is known to be the primary oxidation product of tyrosine residues in proteins and is present in proteins in ageing [20] and disease [19]. This approach has advantages over conventional methods of generating oxidised proteins since the use of strong oxidants such as paraquat [26] or hydrogen peroxide [27] can cause extensive damage to other biological molecules such as lipids and DNA [1,17]. Using the incorporation approach we can generate a precise pool of proteins containing oxidised amino acids without damage to other molecules and can thereby preserve cell viability. To do this we supplied DOPA to the cells in tyrosine-free medium so that DOPA could more effectively compete with tyrosine (present in the cell and in the foetal calf serum) for charging by tyrosyl tRNA synthetase and insertion into the polypeptide chain of proteins [16,25]. The presence of DOPA and DOPA-containing proteins had no effect on cell viability at the levels we used (Figure 3B). Incorporation of DOPA into proteins results in the generation of non-native/oxidised proteins in cells and thus might equate to a period of acute oxidative stress or an inflammatory insult *in vivo*. When DOPA-containing proteins were present in cells, both young and aging cells degraded these proteins much more rapidly than native ($^{14}$C leucine labeled) proteins (Figure 4A & B) proving evidence that the cells recognise at least some of the DOPA-containing proteins as damaged and selectively degraded them. We have previously demonstrated that degradation of DOPA-containing proteins by cells is biphasic, and at higher levels of DOPA incorporation, DOPA-containing proteins resist degradation [16] and aggregate [6]. In the present studies, at higher DOPA concentrations, there was a significant decline in the rate of degradation of DOPA-containing proteins in aging cells relative to young cells (Figure 4B). The ability of aging cells to degrade aggregate-prone, DOPA-containing proteins was clearly impaired. The two major pathways for the degradation of oxidized proteins are the proteasome and lysosomes. We showed that there was a reduction in the activity of the proteasome in aging cells compared to young cells where the trypsin-like activity was reduced to 43%, the PGPH to 66% and the

chymotrypsin to 89% (Table 1). The observation of an age-related reduction in proteolytic activity is supported by a large body of evidence [2,30] and this also applies in age-related pathologies [31–35]. A reduction in proteasome activity in aging cells also corresponded to a decline in the activity of the lysosomal enzymes; aryl sulphatase, acid phosphatase and acid lipase (Table 1). This may have been a result of an overall reduction in the population of lysosomes in the ageing cells.

There was a significant reduction in HSP70 protein levels in aging MRC-5 cells before there was any evidence of oxidised protein accumulation (Figure 2A). HSP70 is an inducible cytosolic heat shock protein that rapidly responds to thermal or oxidative stress, to sequester misfolded proteins by its binding domain, and thus preventing damaged proteins from forming aggregates. In this study we showed that HSP70 could be induced in aging cells both by exposure to DOPA or mild heat stress (Figures 5 and 6). Exposure to mild heat stress resulted in a small but significant increase in the rate of degradation of DOPA-containing proteins suggesting that HSP70 played a role in facilitating the degradation of these proteins (Figure 6B).

The accumulation of oxidised proteins in cells and tissues is a common feature of neurodegeneration and other disorders associated with aging. As was evident in the present studies, cell division and the distribution of oxidised material amongst daughter cells is an important mechanism that allows dividing cells to limit the accumulation of oxidised proteins [36,37]. The inability of senescent cells to divide could therefore account for the rapid accumulation of DOPA-containing proteins. This could occur in long-lived cells such as retinal pigment epithelial cells or neurons. Age pigments have also been shown to directly inhibit the function of the proteasomes and the lysosomes leading to a decline in cell function.

Whilst a great deal of research has focused on changes to the proteolytic machinery in aging

and neurodegeneration HSPs, which serve a critical upstream function, have received less attention. HSPs serve several roles in maintaining homeostasis including, protecting against aggregation, solubilizing of early stage protein aggregates, assisting in folding of nascent proteins or in refolding of damaged proteins. Importantly they also target damaged proteins to the degradation machinery and sequester non-degradable proteins to larger aggregates [12].

## Conclusions

By experimentally generating oxidised proteins in cells we showed that the basal turnover of cell proteins was maintained in ageing MRC-5 cells and they were able to efficiently degrade low levels of oxidised proteins. When higher levels of oxidised proteins were experimentally generated however their degradation in ageing cells was less efficient than in young cells. Impairment in proteasome activity and a decline in HSP function appeared to contribute to the decline in oxidised protein turnover. HSP70 levels were reduced in ageing cells but could be increased by mild heat stress resulting in an increased ability to degrade oxidise proteins. These studies describe a useful *in vitro* model to study the mechanisms of degradation of damaged or non-native proteins and highlight the importance of heat shock proteins in the removal of oxidized proteins and thus, the maintenance of homeostasis in aging and age-related pathologies.

## Acknowledgements

This work was supported by an NHMRC Australia project grant 189800.

**Legends to Figures**

**Table 1:** The peptidase activity of the proteasome (chymotrypsin-like, trypsin-like and PGPH activities), the lysosomal proteases (cathepsins B, L and S) and activity of lysosomal arylsulphatase, acid phosphatase and acid lipase in aging (PD36) MRC-5 cells and young (PD16) cells was compared. Activity was measured using fluorescently labelled substrates and set at 100% for young cells. Results represent triplicate sets of cultures and data was expressed as change in fluorescence per minute per mg of protein. * $p < 0.05$, ** $p < 0.01$, errors represent SEM.

**Figure 1:** A. Accumulation of protein-bound DOPA in ageing MRC-5 fibroblasts. DOPA in hydrolysed cell proteins was measured by HPLC and expressed as a ratio to tyrosine. B. At PD42 MRC-5 cells entered post-mitotic senescence. Cells were maintained in culture for more than 3 months after reaching post-mitotic senescence and native autofluorescence imaged using an inverted Olympus IX71 microscope with a NIBA filter set (ex 470–490nm, em 515–550nm) equipped with a DP71 digital camera with exposure of 0.22 seconds.

**Figure 2:** Densitometric analysis of HSP levels in MRC-5 cell lysates identified by western blotting. Cells were examined between PD13 and PD40 for levels of: A. HSP70, B. HSP90 and C. HSP27. Relative expression was quantified by densitometry (see insets). Statistically significant changes in the HSP protein levels relative to the young cells (PD16) were as indicated, ** $p < 0.01$, *** $p < 0.001$ and errors represent SEM.

**Figure 3:** A. Incorporation of DOPA into cell proteins from young (PD16) and aging (PD36) MRC-5 cells was measured. After incubation with DOPA (0 to 750μM) for 24 hours levels of DOPA in hydrolysed cell proteins was measured by HPLC (expressed as mMoles of DOPA per Mole tyrosine). B. The effect of DOPA on viability of young (PD16) and aging (PD36) cells was measured by quantifying LDH release from cells after incubation with DOPA for 24 hours. No decrease in cell viability was detected.

**Figure 4:** A. The degradation rate of $^{14}$C-leucine labelled (bulk) proteins was measured in young (PD16) and aging (PD36) MRC-5 cells over an 8 hour period. This was expressed as a percentage of the total pool of $^{14}$C-leucine labelled proteins generated in the preceding 24 hours. B. The degradation of $^{14}$C-DOPA labelled (oxidised) proteins was measured in young (PD16) and aging (PD36) MRC-5 cells over an 8 hour period. This was expressed as a percentage of the total pool of $^{14}$C-DOPA labelled proteins generated in the preceding 24 hours, * $p < 0.05$, **$p < 0.01$ and errors represent SEM.

**Figure 5:** Densitometric analysis of western blots of HSP70 protein levels in aging MRC-5 cells (PD36) exposed to DOPA (500μM) in tyrosine-depleted EMEM for 24 hours (Dark bars) compared to MRC-5 cells incubated in tyrosine-depleted EMEM for 24 hours without DOPA (Light bars). Relative expression of HSP27, HSP70 and HSP90 were measured by western blot and quantified by densitometry. Data are expressed relative to HSP protein levels in young cells (PD16). Results represent triplicate sets of cultures * $p < 0.05$ and error bars represent SEM.

**Figure 6:** A. Western blotting analysis of HSP70 protein levels in aging MRC-5 cells (PD36) exposed to mild heat stress (42˚C for 15 mins). B. MRC-5 cells (PD36) that had been pre-exposed to mild heat stress (labelled as mild stress) or control cells that were not exposed to mild heat stress (labelled control) were incubated with $^{14}$C-DOPA (5 μM) and increasing concentrations of non-labelled DOPA to give final concentrations 50 to 750 μM as indicated. The degradation rate of $^{14}$C-DOPA-labelled proteins was then measured over the following 8 hours in tyrosine saturated EMEM. Results represent triplicate sets of cultures ***$p < 0.001$ and errors represent SEM.

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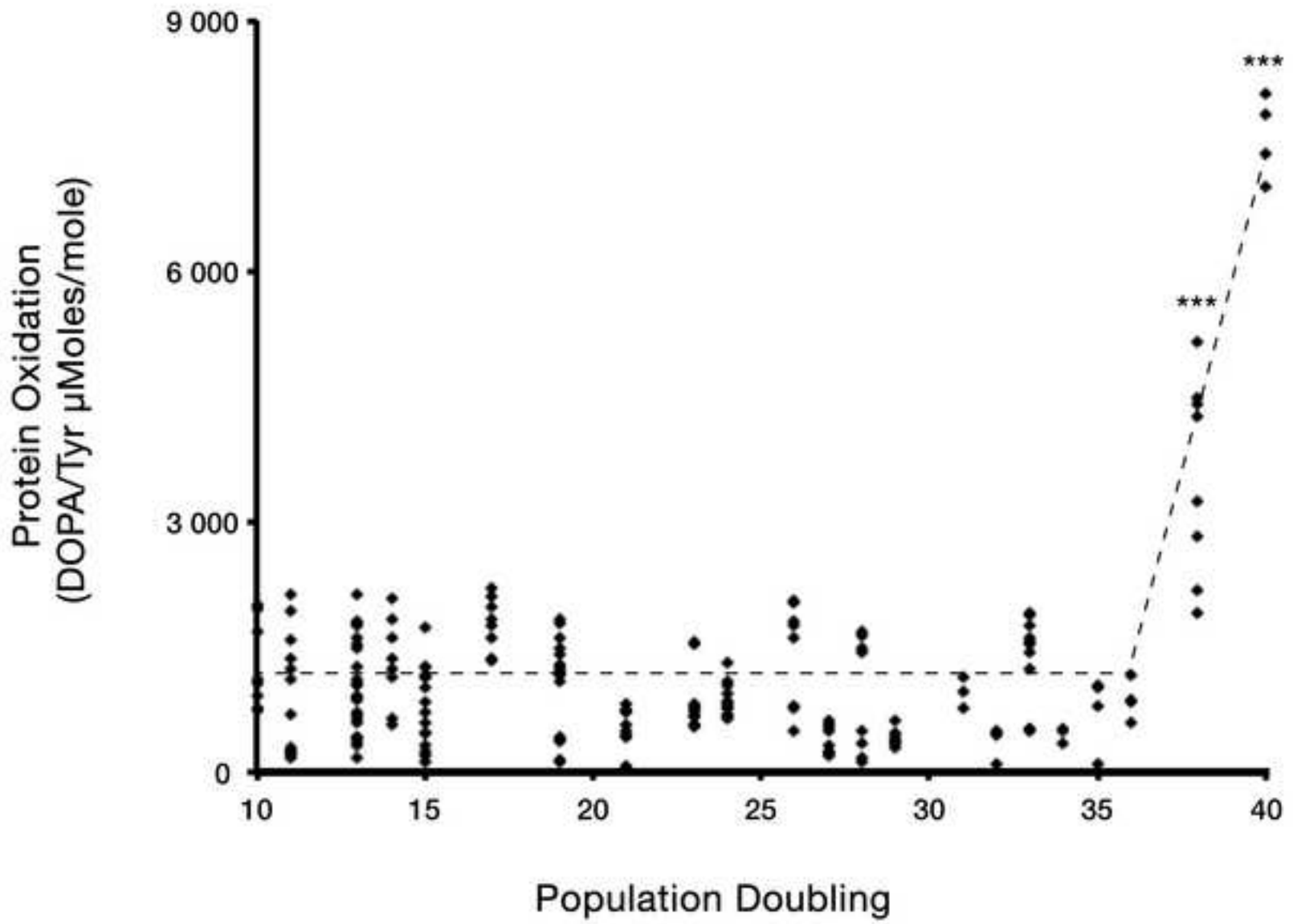
**Table 1**

| | Proteasome activity | | |
|---|---|---|---|
| | Chymotrypsin-like | PGPH | Trypsin-like |
| Young (PD16) | 100 + 5.7 | 100 + 2.0 | 100 + 17.2 |
| Aging (PD36) | 88.9 + 0.7 | 66.1 + 4.4** | 42.8 + 6.6* |

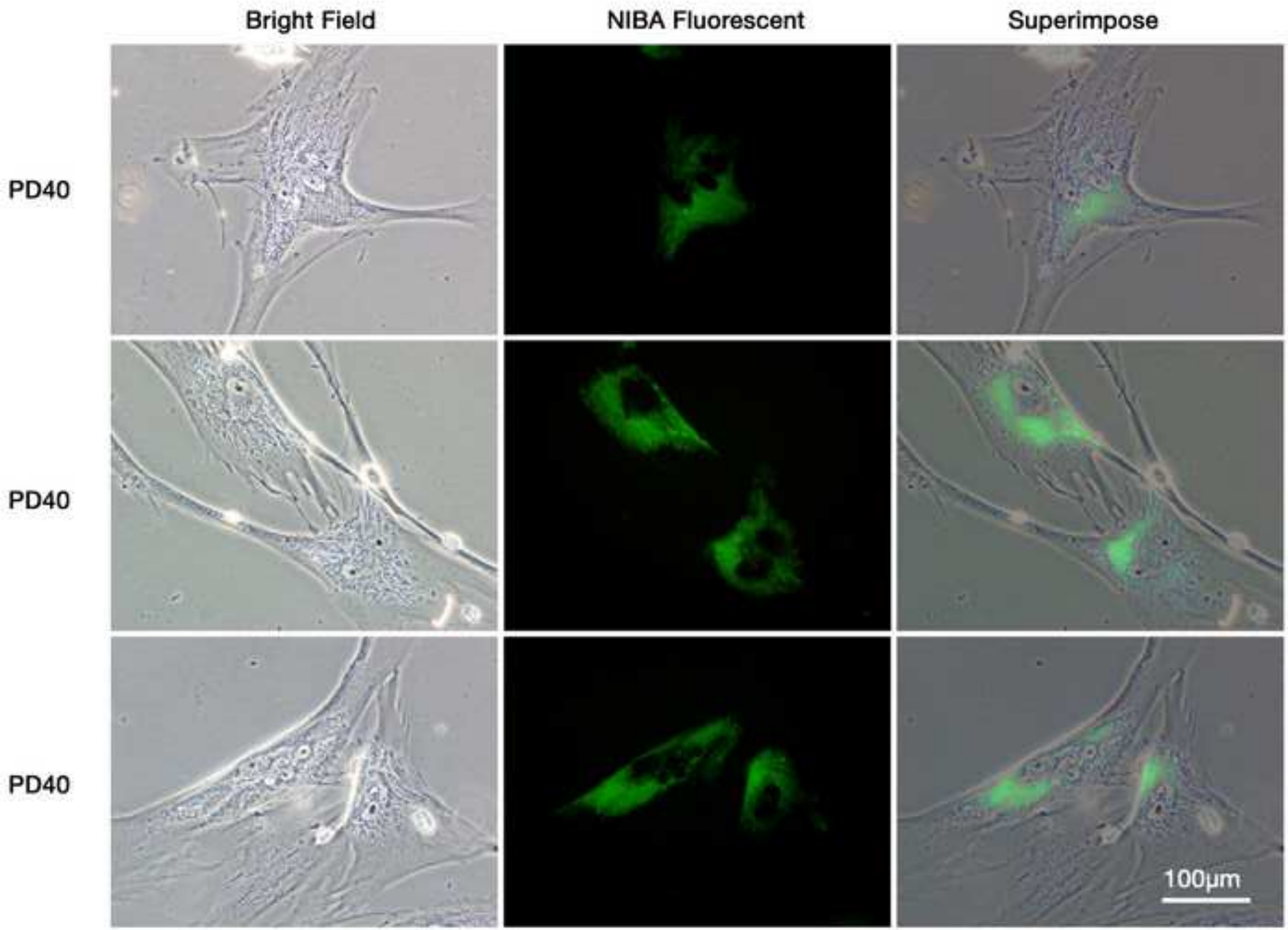
| | Lysosomal protease activity | | |
|---|---|---|---|
| | Cathepsin L | Cathepsin B | Cathepsin S |
| Young (PD16) | 100 + 17.5 | 100 + 16.6 | 100 + 30.1 |
| Aging (PD36) | 72 + 8.5 | 71.1 + 8.17 | 57.4 + 5.2 |

| | Lysosomal enzyme activity | | |
|---|---|---|---|
| | Arylsulphatase | Acid phosphatase | Acid Lipase |
| Young (PD16) | 100 + 1.4 | 100 + 0.02 | 100 + 12.1 |
| Aging (PD36) | 88.2 + 1.7** | 83.9 + 0.04** | 56.3 + 6.2* |

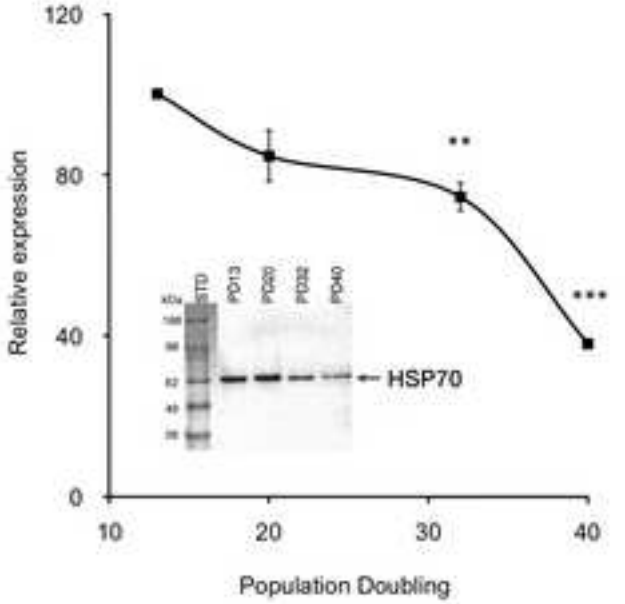
Figure

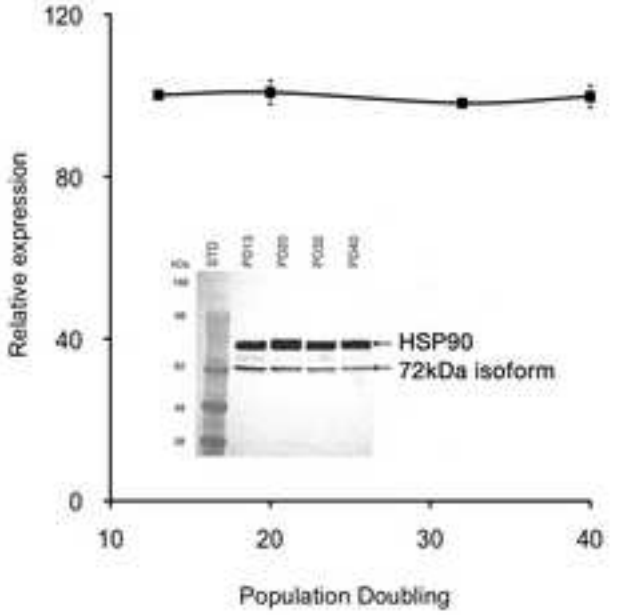

9 000
6 000
3 000
0
Protein Oxidation
(DOPA/Tyr µMoles/mole)
***
***
10
15
20
25
30
35
40
Population Doubling

Figure


Bright Field
NIBA Fluorescent
Superimpose
PD40
PD40
PD40
100μm

**Figure**


120
80
40
0
10
20
30
40
Relative expression
Population Doubling
**
***
STD
PD13
PD20
PD32
PD40
HSP70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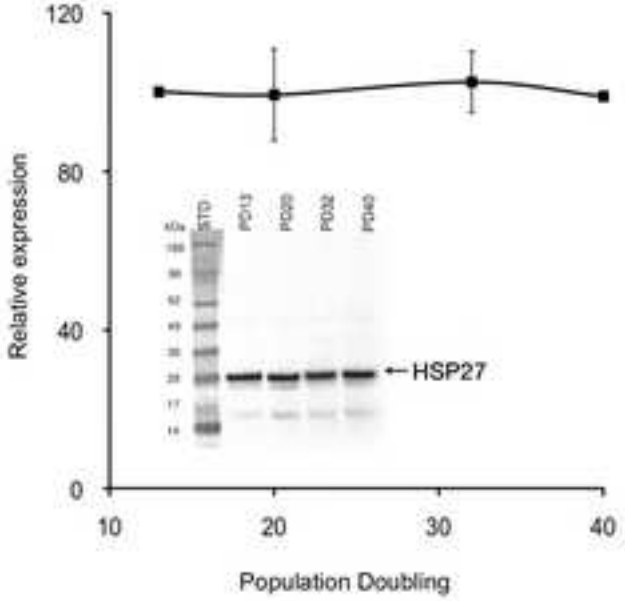
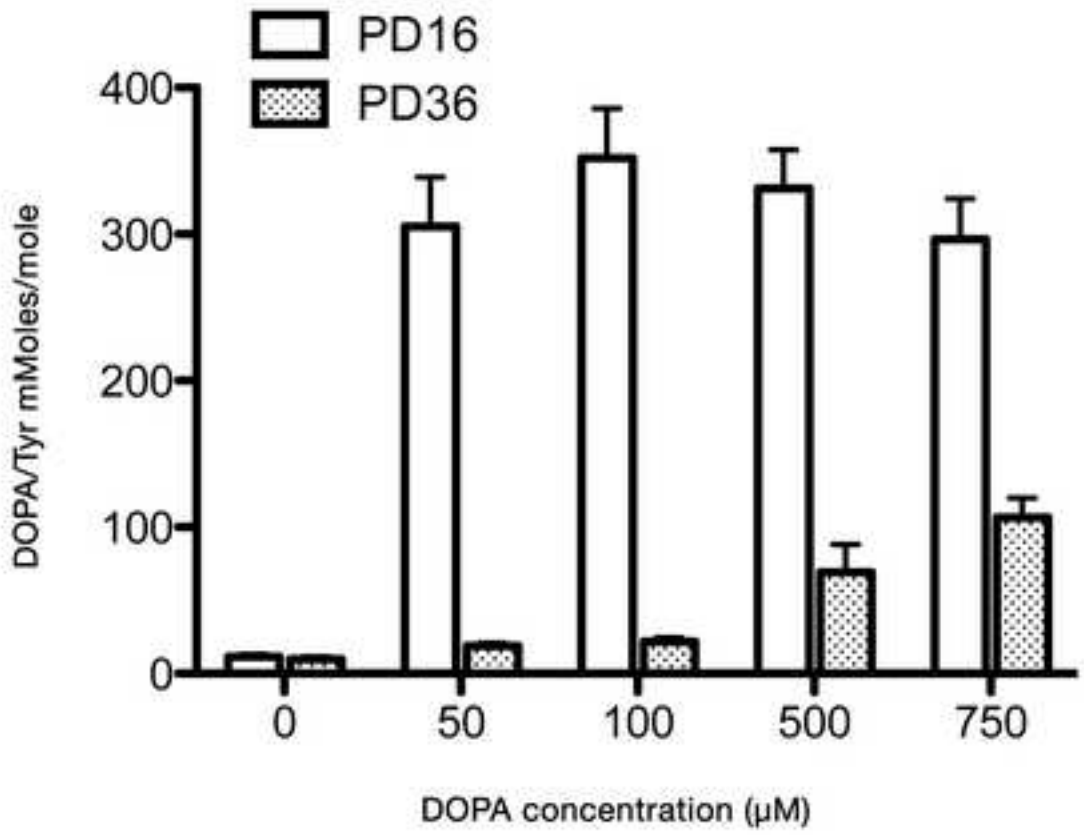
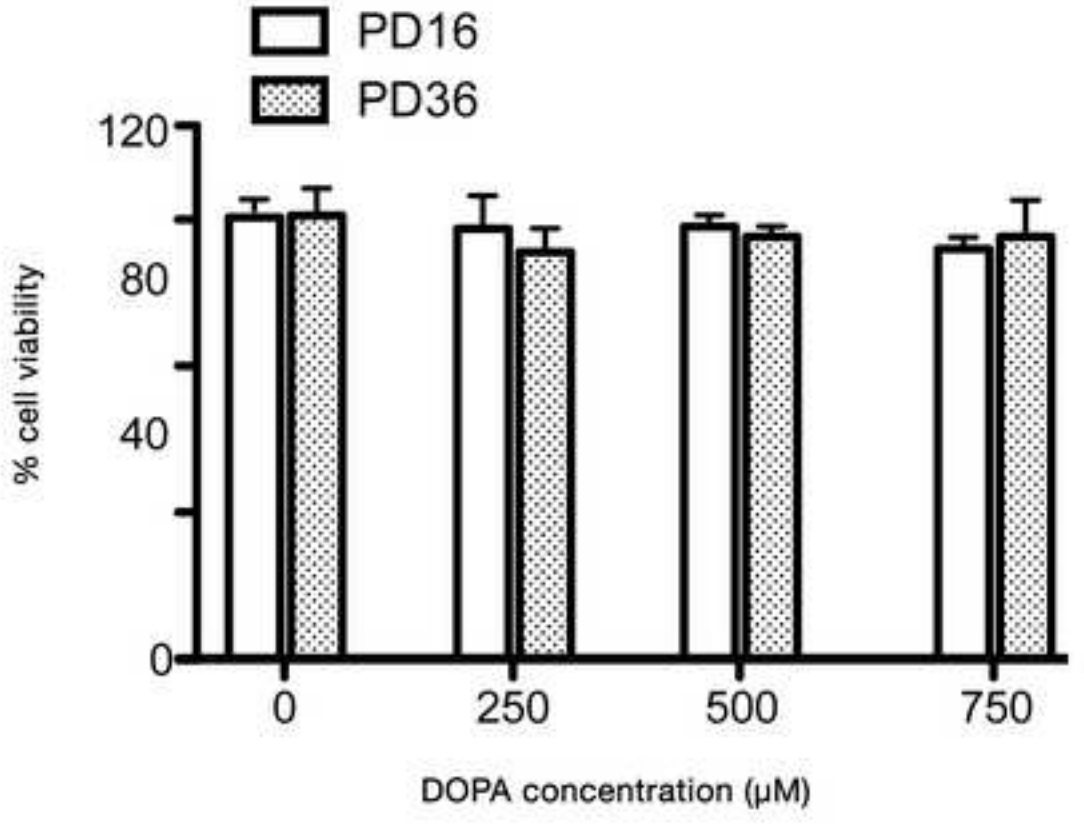
40
0
10
20
30
40
Relative expression
Population Doubling
STD
HSP90
72kDa isoform

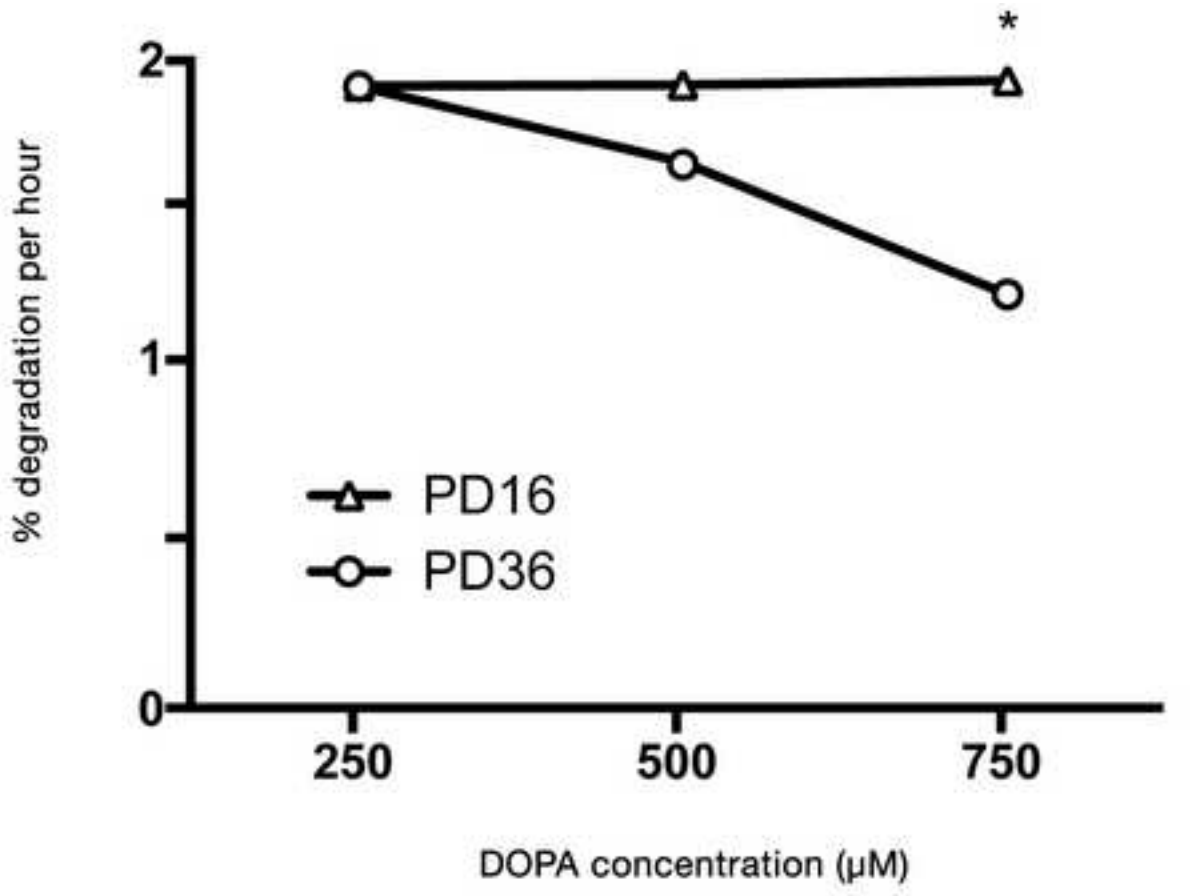

120
80
40
0
10
20
30
40
Relative expression
Population Doubling
STD
PD13
PD20
PD32
PD40
HSP27

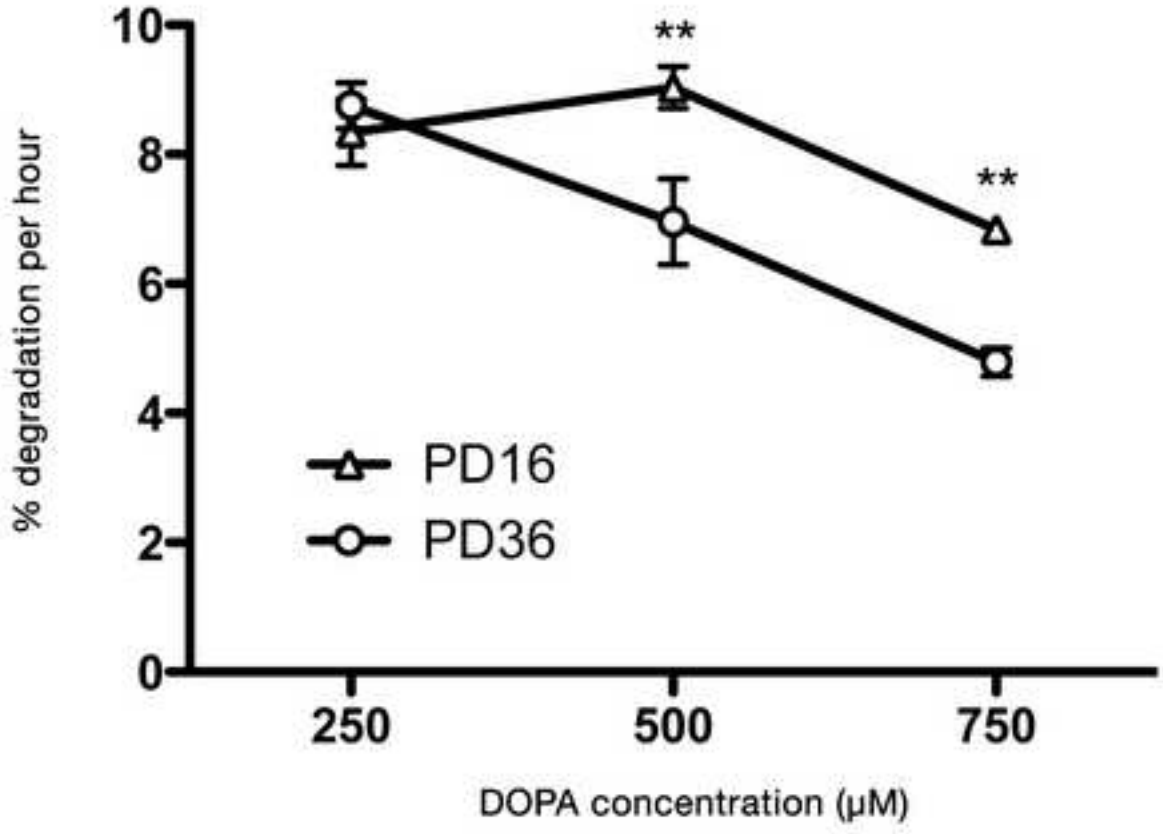
Figure


PD16
PD36
400
300
200
100
0
DOPA/Tyr mMoles/mole
0
50
100
500
750
DOPA concentration (μM)

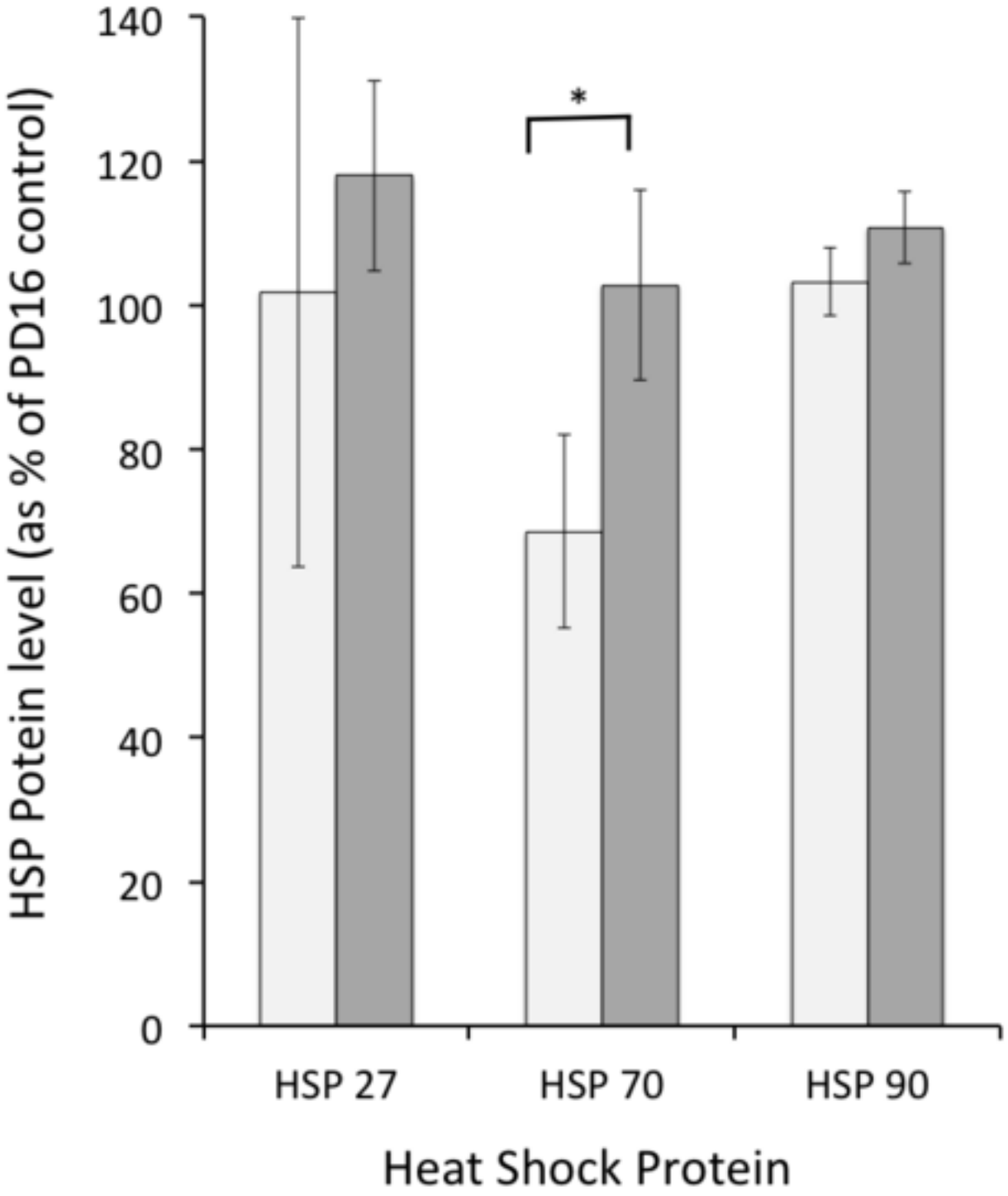

PD16
PD36
120
80
40
0
% cell viability
0
250
500
750
DOPA concentration (μM)

Figure

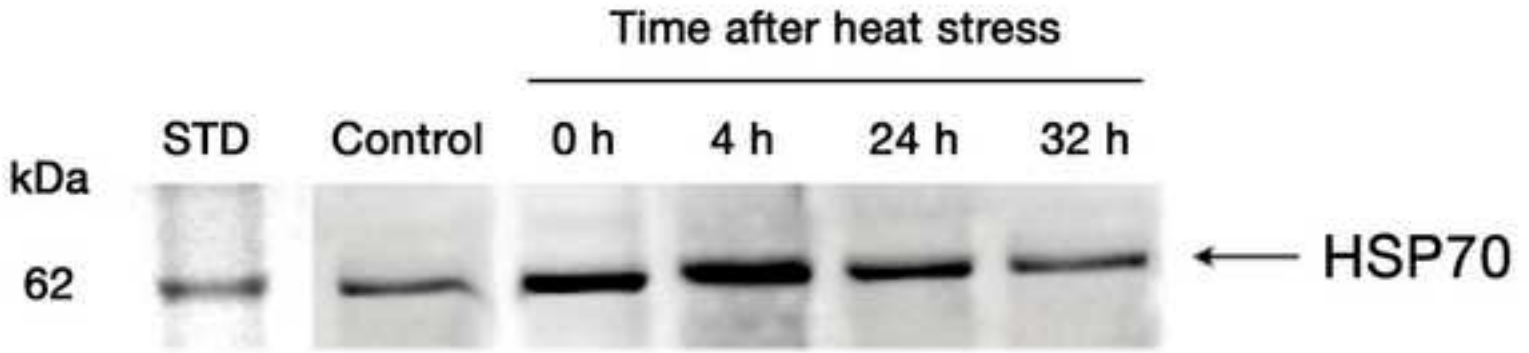

*
2
1
0
% degradation per hour
PD16
PD36
250
500
750
DOPA concentration (μM)


**
**
10
8
6
4
2
0
% degradation per hour
PD16
PD36
250
500
750
DOPA concentration (μM)

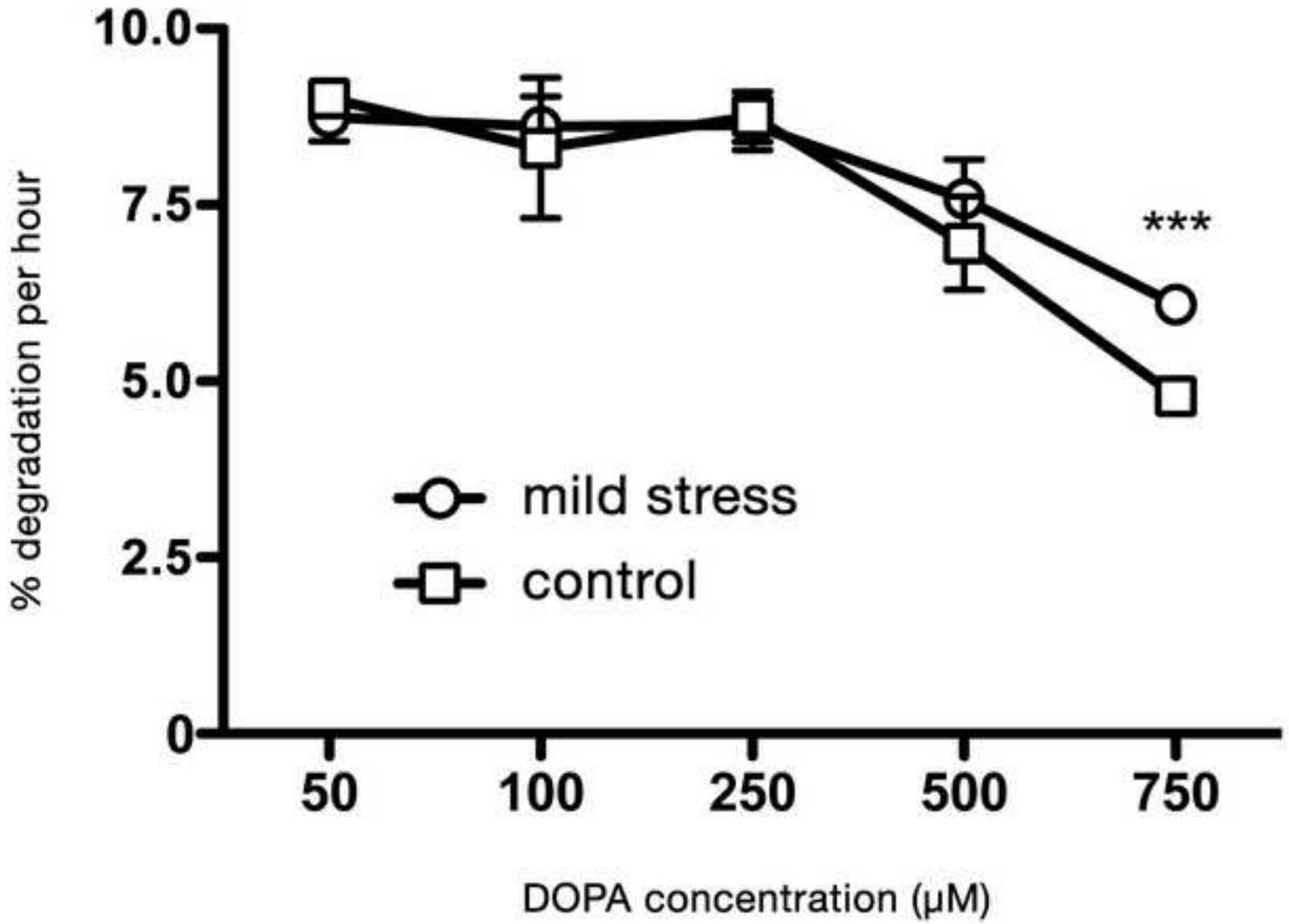
Figure


140
120
100
80
60
40
20
0
HSP Potein level (as % of PD16 control)
*
HSP 27
HSP 70
HSP 90
Heat Shock Protein

Figure



Figure


10.0
7.5
5.0
2.5
0
% degradation per hour
***
mild stress
control
50
100
250
500
750
DOPA concentration (μM)