

# Chaperone function and chaperone overload in the aged. A preliminary analysis

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## Abstract

Chaperones have an important role in the repair of proteotoxic damage, which is greatly increased in aged subjects. Chaperone levels and expression were subject of numerous studies in aged organisms. However, there were only very few attempts to measure chaperone activity in aged animals. Here, we report our initial studies showing a decreased chaperone capacity of liver cytosol from aged rats compared to those of young counterparts. The amount of Hsc70/Hsp70 was not significantly different in livers of young and aged rats. On the contrary, old animals showed a significant decrease in their hepatic Hsp90 content, which may explain their decreased chaperone activity. The observed decrease in chaperone capacity may also reflect a direct proteotoxic damage of chaperones, or an increase in chaperone occupancy, i.e. a 'chaperone overload' due to the increased amount of damaged hepatic proteins in aged rats. Experiments are in progress to elucidate the mechanism of the observed age-induced changes in chaperone function. © 2002 Elsevier Science Inc. All rights reserved.

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## 1. Introduction

Chaperones are ubiquitous, highly conserved proteins, which either assist in folding of newly synthesized or damaged proteins in an ATP-dependent, active process or work in an ATP-independent, passive mode sequestering damaged proteins for future refolding or digestion (Bukau and Horwich, 1998; Hartl, 1996). Chaperones are especially needed and their synthesis is induced after an environmental stress leading to proteotoxic damage.

Aging is characterized by an increased rate of protein modification such as oxidation, glycation, deamidation of asparaginyl and glutaminyl residues and the subsequent formation of isopeptide bonds, etc. (Stadtman and Berlett, 1998; Wright, 1991). Susceptibility to various proteotoxic damages is further increased due to transcriptional and translational errors and the resulting folding defects (Dukan et al., 2000).

Due to the decrease in proteasome function during aging (Conconi et al., 1996; Bulteau et al., 2001) as well as the impaired lysosomal protein degradation in aged rats (Cuervo and Dice, 2000), damaged proteins accumulate in cells of aged and may cause a chaperone overload. Here, the competition of

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damaged proteins may disrupt the folding assistance of other chaperone targets, such as: (1) newly synthesized proteins; (2) ‘constantly damaged’ (mutant) proteins; and (3) constituents of the cytoarchitecture (Csermely, 2001a,b).

Despite of the large number of studies on chaperone levels and induction in aged organisms (reviewed by Söti and Csermely (2002) and Verbeke et al. (2001)) direct studies on chaperone function in aged organisms are largely restricted to the eye lens chaperone,  $\alpha$ -crystallin. Chaperone activity of  $\alpha$ -crystallin is decreased in senile human lenses (Cherian and Abraham, 1995). As another of the sporadic examples of chaperone function in aged animals, Hsp90 fails to protect the proteasome in aged animals (Conconi et al., 1996).

In our present studies we have pursued an initial test of passive chaperone function by an indirect method, by measuring the attenuation of heat-induced luciferase denaturation by liver cytosolic preparations from young and aged rats. Our data show a decreased chaperone capacity of liver cytosol from aged rats compared to those of young counterparts, which is the first data on total chaperone function of cytosolic chaperones in aging.

## 2. Materials and methods

### 2.1. Materials

Anti-Hsc/Hsp70<sup>1</sup> antibody (rabbit, polyclonal) was a kind gift of Zoltán Péntzes (Biorex R&D Co., Veszprém, Hungary) (Kurucz et al., 1999). Anti-Hsp90 $\alpha/\beta$  antibody (goat, polyclonal, sc-1055) was a Santa Cruz (Santa Cruz, CA, USA) product. Anti-luciferase antibody (goat, polyclonal) was purchased from Promega (Madison, WI, USA). Secondary anti-rabbit, and anti-goat antibodies were DAKO A/S products (Glostrup, Denmark). Chemicals used for polyacrylamide gel electrophoresis and protein determination were from Bio-Rad (Richmond, CA, USA). All other chemicals (including luciferase) used were from Sigma Chemicals Co. (St Louis, MO, USA).

<sup>1</sup> Abbreviations used: Hsc70, the constitutive form of the 70 kDa heat shock protein; Hsp70, inducible form of the 70 kDa heat shock protein; Hsp90, 90 kDa heat shock protein.

### 2.2. Animals

Young (10 weeks  $\pm$  3 days old) and aged (26 months  $\pm$  2 weeks old) Wistar rats were from Charles River Inc. (Hungary). Animals were kept and sacrificed according to the Guidelines of the Hungarian Council of Health Sciences (permission no. 39/1999).

### 2.3. Isolation of rat liver cytosol

Livers were removed and homogenized by a Potter–Elvehjem homogenizer in two volumes of an ice-cold buffer consisting of 20 mM Hepes, pH 7.4 and a complete protease inhibitor cocktail (Roche, Mannheim, Germany). Liver homogenates were filtered through a cheesecloth and centrifuged at 4 °C for 10 min at 700  $\times$  g. Supernatants were centrifuged in a Beckman J2-HS centrifuge at 4 °C for 10 min at 12,000  $\times$  g. Postmitochondrial supernatants were cleared from microsomes in a Beckman Optima TL ultracentrifuge using a TLA 100.4 rotor at 4 °C for 60 min at 100,000  $\times$  g. The supernatant cytosol was immediately aliquoted, frozen in liquid nitrogen and stored at  $-80$  °C. Extreme care was exercised to use the thawed aliquots immediately and never re-freeze them. Protein content of the obtained cytosolic samples was measured using the Bradford (1976) method with bovine serum albumin as standard.

### 2.4. Protection of luciferase from heat denaturation by cytosolic chaperones

Cytosolic proteins at a final concentration of 40 mg/ml were mixed with 400 nM of firefly luciferase in a final volume of 200  $\mu$ l of a buffer consisting of 20 mM Hepes, pH 7.5 and 50 mM potassium acetate and incubated at 39 °C. At time points indicated 2  $\mu$ l aliquots were removed from the incubation mixture, and their luciferase activity was measured by adding them to 36  $\mu$ l of the reaction mixture containing 25 mM Tricin (pH 7.8), 10 mM MgSO<sub>4</sub>, 0.2 mM EDTA, 20 mM DTT, 0.26 mM Coenzyme A, 1 mM ATP and 30  $\mu$ g/ml luciferin. Luciferase activity was measured in a BioOrbit Galaxy 1258 luminometer (Turku, Finland) with an integration time of 10 s, at a normal gain setting.

### 2.5. Protection of luciferase from aggregation by cytosolic chaperones

Cytosolic proteins at a final concentration of 40 mg/ml were mixed with 400 nM of firefly luciferase in a final volume of 200  $\mu$ l of a buffer consisting of 20 mM Hepes, pH 7.5 and 50 mM potassium acetate and incubated at temperatures indicated. At time points indicated all the 200  $\mu$ l samples were removed from the thermostated tubes, and centrifuged in a Beckman Optima TL ultracentrifuge using a TLA 100.1 rotor at 4 °C for 10 min at 350,000  $\times$  g. Supernatants were carefully removed. Luciferase content of the supernatants and ice-cold control samples without incubation/centrifugation procedures were assessed by subjecting the samples of SDS PAGE and consecutive Western blotting. Blots were visualized by anti-luciferase antibody using the ECL chemiluminescence kit.

### 2.6. Assessment of chaperone levels

80  $\mu$ g of cytosolic proteins were subjected to SDS PAGE on a 10.5% gel using a BioRad MiniProtein B system. Gels were blotted with a home-made semidry blotting apparatus to Protran nitrocellulose membrane (Schleicher & Schuell Co., Keene, NH, USA). Blots were blocked by 2% bovine serum albumin and visualized by anti-Hsc/Hsp70 and anti-Hsp90 $\alpha$ / $\beta$  antibodies using the ECL chemiluminescence kit. Loading efficiency was controlled by assessing the identical amount of the externally added luciferase to the samples. Photographic images were quantitated by densitometry using an LKB Ultrascan XL laser densitometer (Bramma, Sweden).

### 2.7. Statistical evaluation

Statistical evaluation of luciferase heat denaturation data was performed by the Student's *t*-test.

## 3. Results

Parallel with our ongoing experiments to measure the active (ATP-dependent) chaperone activity of cytosolic preparations, we also assessed the passive (ATP-independent) chaperone activity of liver

cytosols using an indirect method. Here, we report our initial results of these, latter experiments. For this we had to find a test protein, which is much more susceptible to denaturation than most of the cytosolic proteins, including molecular chaperones. Luciferase, a commonly used firefly enzyme was suitable for this purpose, since it rapidly loses its activity when incubated at 39 °C, where most of cellular proteins (potential other targets and chaperones) still remain intact (Freeman et al., 2000).

The effect of cytosol from young and old rats on the heat-induced luciferase denaturation is shown in Fig. 1. Luciferase denaturation is complete in 30 min irrespective of the presence of protecting cytosolic chaperones. At all other time points measured cytosol from livers of young (10 weeks old) rats shows a better protection than that of old (26 months old) animals. The difference between the protected luciferase activity is significant after 5 min of incubation reaching a level of significance of  $p < 0.03$ .

Another important consequence of ATP-independent chaperone function is the prevention of protein aggregation. Therefore, a different test of passive

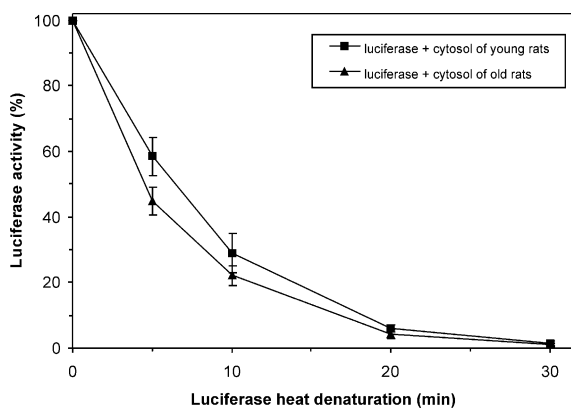


Fig. 1. Protection of luciferase activity against heat denaturation by cytosol of young (10 weeks) and old (26 months) Wistar rats. Isolation of rat liver cytosol, incubation of luciferase and measurement of luciferase activity were performed as described in Section 2. Squares: luciferase activity in the presence of cytosol from young (10 weeks) rats. Triangles: luciferase activity in the presence of cytosol from old (26 months) rats. Data were obtained from duplicate measurements from three rats per group. There is a significant difference between luciferase activity in the presence of cytosol from young and old rats at 5 min of incubation with a level of significance  $p < 0.03$ .

chaperone function giving complementary results to the experiments shown in Fig. 1, is the assessment of the heat-induced aggregation of luciferase. In initial experiments, we obtained a measurable aggregation of the enzyme (i.e. partition to the pellet after ultracentrifugation at  $350,000 \times g$  for 10 min) both at 39 and 42 °C, but not at 0 °C, where no aggregation was detected neither in the presence of young nor in the presence of aged cytosols (data not shown). Measurement of luciferase aggregation in the presence of cytosolic proteins from livers of young and old rats at 42 °C showed a larger amount of non-aggregated luciferase in the presence of cytosols from young rats (Fig. 2;  $0.21 \pm 0.04$  and  $0.15 \pm 0.04$  for young and old animals, respectively). However, the difference was not significant ( $p < 0.104$ ). Examination of luciferase aggregation at less stringent conditions (39 °C) did not give conclusive results (data not shown).

As an obvious reason for the weaker luciferase protection of liver cytosol from aged rats, a decreased chaperone content comes to mind. To analyze this possibility we have measured the levels of the two most abundant cytosolic chaperones, Hsc/Hsp70 and Hsp90 $\alpha/\beta$  (Bukau and Horwich, 1998; Hartl, 1996). As it is shown in Fig. 3A, there were no significant differences in the level of Hsc/Hsp70 in cytosol from young and old rats (the antibody we used recognizes both the constitutive and inducible forms of the rat

70 kDa heat shock protein). On the contrary, old animals contained a significantly lower amount of Hsp90 $\alpha/\beta$  than their young counterparts (the antibody we used recognizes both the alpha and beta isoforms of the rat 90 kDa heat shock protein; Fig. 3B;  $0.60 \pm 0.09$  and  $0.26 \pm 0.10$ ; for young and old animals, respectively,  $p < 0.011$ ).

#### 4. Discussion

The most important finding of the present paper is a decreased chaperone capacity of liver cytosol from aged rats compared to those of young animals, which is the first data on total chaperone function of cytosolic chaperones in aging.

The attenuated chaperone function in aged rats might have at least three independent reasons: (1) the amount of chaperones was diminished; (2) chaperones were damaged; (3) chaperones became occupied by an increased amount of damaged proteins or peptides. Analyzing the first possibility, we measured the levels of the two most important cytosolic chaperones, Hsc/Hsp70 and Hsp90. The significant decrease in Hsp90 content may explain the diminished hepatic chaperone capacity of aged rats. Existing data on chaperone levels in livers of aged rats are rather contradictory: Wu et al. (1993) measured an unchanged Hsc70 level, while Cuervo and Dice

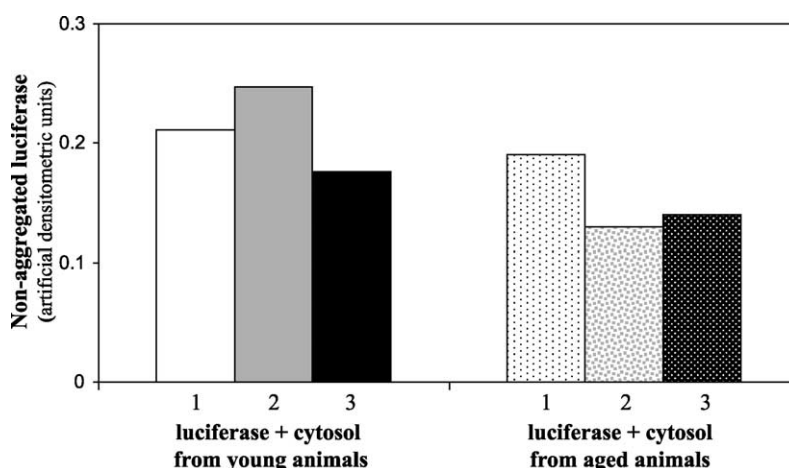


Fig. 2. Prevention of luciferase aggregation by cytosol samples from livers of young (10 weeks) and old (26 months) Wistar rats. Isolation of rat liver cytosol, incubation of luciferase at 42 °C, and measurement of luciferase aggregation were performed as described in Section 2. Bars show the amount of non-aggregated luciferase in the presence of cytosolic proteins of each examined animal in artificial densitometric units.

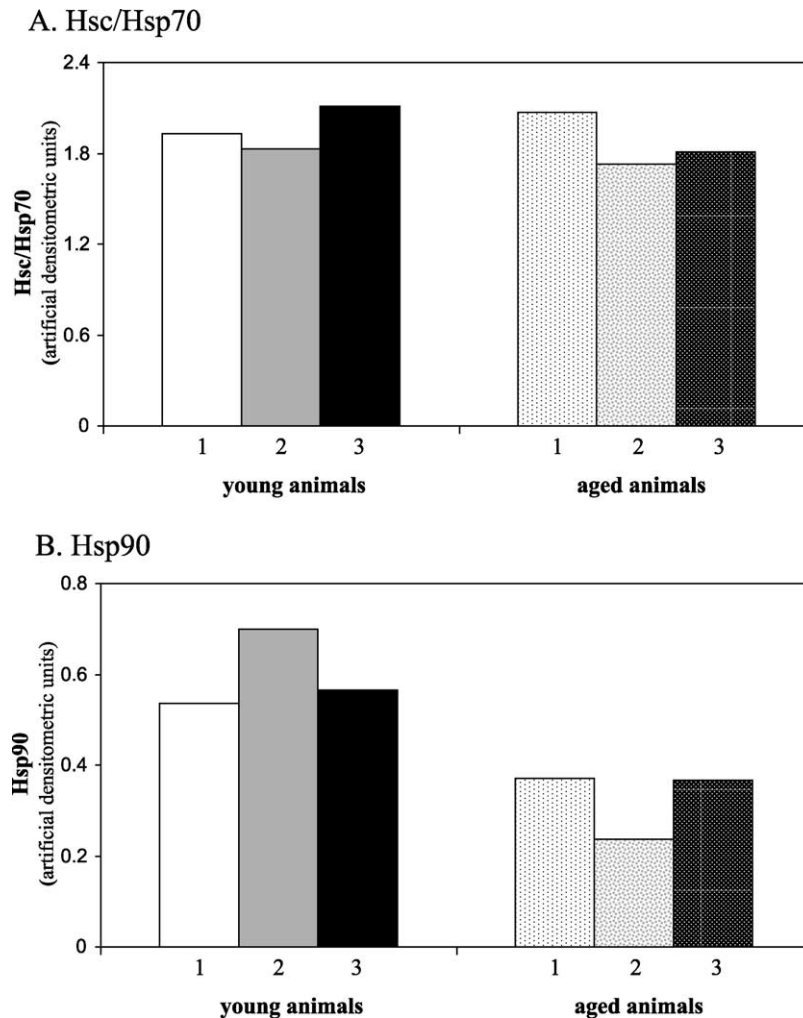


Fig. 3. Level of major cytosolic chaperones from livers of young (10 weeks) and old (26 months) Wistar rats. Isolation of rat liver cytosol, and measurement of Hsc/Hsp70 and Hsp90 $\alpha/\beta$  levels were performed as described in Section 2. Bars show the amount of the respective cytosolic heat shock protein of each rat in artificial densitometric units. Panel A: Hsc/Hsp70 levels. Panel B: Hsp90 $\alpha/\beta$  levels.

(2000) and Hall et al. (2001) showed an increased Hsc70 (and Hsp70) content of old rats, while to our best knowledge no report assessed hepatic Hsp90 levels of old rats. Higher chaperone levels may reflect an adaptation mechanism to the growing number of unfolded polypeptide chains, which titrate out the chaperones from the heat shock factor complex, and induce a constitutive stress response. This rather permanent stress probably ‘wears out’ the mechanism to mobilize the stress response, which may explain

why the *induction* of various chaperones is impaired in aging (Söti and Csermely, 2002). The intrinsic variability of the experienced stress and the various ages and strains used in different studies may explain the observed variability of chaperone levels in aged rats.

The observed decrease in chaperone capacity may also reflect a direct proteotoxic damage of chaperones, or an increase in chaperone occupancy, i.e. a ‘chaperone overload’ due to the increased amount of

damaged hepatic proteins in aged rats. Macario and Conway de Macario (2002) raised the idea of ‘sick chaperones’ in aged organisms in a recent review. Indeed: chaperones are interacting with a plethora of other proteins (Csermely, 2001a), which requires rather extensive binding surfaces. These exposed areas may make chaperones a preferential target for proteotoxic damage: chaperones may behave as ‘suicide proteins’ during aging. On the other hand, the dramatic increase of potential targets, the ‘chaperone overload’ (Csermely, 2001b), saturates the remaining chaperone capacity and worsens the situation probably further. Experiments are in progress to elucidate the mechanism of the observed age-induced changes in chaperone function by analyzing the amount of damaged proteins, and the structure/function relationships of chaperones.

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