Amino Acids (2007) 32: 603–606
DOI 10.1007/s00726-006-0448-1
Printed in The Netherlands

Amino Acids

Protein-carbonyl accumulation in the non-replicative senescence of the methionine sulfoxide reductase A (*msrA*) knockout yeast strain

Short Communication

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Received September 5, 2006
Accepted September 18, 2006
Published online November 3, 2006;

Summary. The major enzyme of the methionine sulfoxide reductase (Msr) system is MsrA. Senescing *msrA* knockout mother yeast cells accumulated significant amounts of protein-carbonyl both at 5 generation-old (young) and 21 generation-old (old) cultures, while the control mother cells showed significant levels of protein-carbonyl mainly in the old culture. The Msr activities of both yeast strains declined with age and exposure of cells to H_2O_2 caused an accumulation of protein-carbonyl especially in the *msrA* knockout strain. It is suggested that a compromised MsrA activity may serve as a marker for non-replicative aging.

Keywords: Oxidative-stress – Non-replicative senescence – Post-translation modification

Introduction

Accumulative posttranslational modification to proteins, mediated by the action of reactive oxygen species (ROS), is thought to be one of the major causes of aging and age-related diseases. Thus, mechanisms have evolved to prevent or reverse these protein modifications. While most protein damage by ROS is irreversible, methionine oxidation (MetO) to proteins can be reversed by the methionine sulfoxide reductase system (consists of MsrA which reduces *S*-MetO and MsrB which reduces *R*-MetO, thioredoxin reductase, thioredoxin, and NADPH) (Moskovitz et al., 2000, 2002a). The action of the Msr system may prevent irreversible protein damage (e.g. protein carbonylation), contribute to the cellular antioxidant resistance, and as a consequence extend organism's life span. An evidence for the possible functions of the Msr system is demonstrated by the adverse effects shown in several organisms lacking the MsrA protein (Moskovitz et al., 1995, 1997, 2001; Romero et al., 2004). Furthermore, overexpression of MsrA in human T cells, yeast, plant, and flies protects them from oxidative stress toxicity, and has demonstrated a 2-fold increase in the life span of flies (Moskovitz et al., 1998; Romero et al., 2004; Ruan et al., 2002). A major biological role of the Msr system is suggested by the fact that the $MsrA^{-}/^{-}$ mouse is more sensitive to oxidative stress, accumulates higher levels of carbonylated protein, and has a shorter life span (by ~40%) than wild-type mice (Moskovitz et al., 2001). Abolishing the *msrA* gene in yeast caused enhanced accumulation of MetO (both as a free amino acid and protein-bound), while shortening their survival rate in response to oxidative stress (Moskovitz et al., 1997, 1998). Recently, the budding yeast *Saccharomyces cerevisiae* has been extensively investigated as a model organism for aging research.

Yeast consists of constantly proliferating cells in which their reduced level of replication is associated with replicative aging. The life span of yeast is reflected in the number of daughter cells produced by a single mother cell (Mortimer and Johnston, 1959). The ability of yeast cells to produce daughter cells declines as a function of their age in the cell culture (Jazwinski, 1999), while the age of the mother cell increases with each cell division. The cells and their nucleolus sizes are increased as a function of age and they become sterile (Mortimer and Johnston, 1959; Sinclair and Guarente, 1997). The causes for the mother cell arrest are yet to be determined. The median replicative life span of most *S. cerevisiae* cells is about 25 generations and the maximum life span is about 40 generations

(Jazwinski, 1993). In light of these observations, it is important to know whether non-replicative aging of the *msrA* knockout yeast strain will demonstrate elevated levels of protein-carbonyl, which is considered to be one of the markers for oxidative stress-related aging (Moskovitz et al., 2002b).

Materials and methods

Materials

Dabsyl-chloride was purchased from Pierce and hydrogen peroxide was purchased from Fisher.

Yeast strains

The *Saccharomyces cerevisiae* strain lacking the *msrA* gene (*Matα ΔmsrA::URA3 his6 leu2*), denoted as MT, was made according to previously described method by (Moskovitz et al., 1997). The corresponding parental strain (*Matα ura3–52 his6 leu2*), denoted as WT, was a gift by Alan Hinnebusch (National Institutes of Health, Bethesda, MD).

Yeast growth and exposure to hydrogen peroxide (H_2O_2)

Yeast cells were grown to late log phase and then inoculated into liquid YPD media (2% glucose, 1% yeast extract, 2% peptone in water) in the presence or absence of 2 mM of H_2O_2 at 30 °C. When cell density reached early log phase ($A_{600} = 0.3$), the cells were harvested, washed three times with PBS, and homogenized by a cell bead-beater using glass beads. Following centrifugation, the resulting supernatants of the cell extracts were used for further analysis.

Isolating young and old yeast cells by elutriation

The method for the isolation of young and old yeast mother cells depends on cell size as previously described by Laun et al. (2001). Cells were separated according to their diameter using the Beckman elutriation system and rotor JE-6B with a standard elutriation chamber. First, wild-type and *msrA* knockout yeast were grown in YPD medium at 30 °C into either mid-logarithmic phase culture ($A_{600} = 1.0$) for isolating mother cells with an average life span of ~5 generation (young) or into stationary phase (one week of growth) for isolating mother cells with an average life span of ~21 generations (old). Following the growth, the resulting cells were harvested and resuspended in PBS buffer. Young and old mother cells (at about 5 and 21 generations, respectively) were isolated by elutriation. The elutriation chamber was loaded with 10^9 cells for each separation. Cells with different diameters were separated into five fractions (I–V). The chamber was loaded at a flow rate of 10 ml/min and a rotor speed of 3200 rpm to elutriate cells with a diameter <5 μm (fraction I). To collect fraction II (diameter 5–7 μm), the flow rate was set to 15 ml/min and rotor speed to 2700 rpm. Fraction III (diameter 7–8.5 μm) was elutriated at 2400 rpm; fraction IV (diameter 8.5–10 μm) at 2000 rpm; and fraction V (diameter 10–15 μm) at 1350 rpm. Fraction V was used as the representative source for the mother cells of both the young and old cultures. The quality of separation of fraction V from the other fractions was verified microscopically.

Following the final separation step, the corresponding cells were stored at −80 °C.

Determination of Msr activity

The ability of the cellular Msr activity to reduce protein-MetO was assayed using a mixture of 200 μM dabsyl-methionine sulfoxide as substrate, 20 mM DTT, 25 mM Tris-HCl (pH 7.0), and yeast protein extract. Following incubation for 30 min at 37 °C, the reaction mixture was monitored for the formation of dabsyl-methionine using an HPLC-reverse phase column (C-18) separation, as previously described by (Moskovitz et al., 1997).

Determination of cellular protein-carbonyl

Disrupted yeast cell extracts were monitored for their protein-carbonyl content by measuring the soluble protein fraction for dinitrophenol binding, using spectrophotometric measurements as previously described by (Starke-Reed and Oliver, 1989). The protein-carbonyl assay was performed according to a carbonyl-assay kit that is based on this procedure and manufactured by Cayman Chemical (Ann Arbor, MI). Additionally, the treated protein extracts were also subjected to western blot analysis using antibodies against DNPH (Oxyblot, Chemicon) for protein-carbonyl detection.

Results and discussion

Non-replicative senescence in yeast is evaluated by isolating old mother cells through the yeast cell generations. The wild-type yeast cell does not show many signs of aging up to 8 cell divisions/generations (McMurray and Gottschling, 2003). Accordingly, 5 and 21 generation-old cells were isolated representing young and old mother cells, respectively. Previously, it was shown that *msrA* null mutant yeast cells (MT) had a shorter life span due to oxidative stress-mediated damage (Moskovitz et al., 1997, 1998). Moreover, an MsrA-overexpressed strain was more resistant to oxidative stress with a slightly better survival rate than in its respective wild-type strain (WT) (Moskovitz et al., 1998). These observations suggest that the MT strain is more prone to oxidative damage to proteins resulting in shorter life span. To investigate the possibility that this MT strain also accumulates non-reversible posttranslational modifications with age, its protein-carbonyl levels were monitored in 5 and 21 generation-old cells relative to its control WT strain. As shown in Fig. 1A, the level of protein-carbonyl in the *msrA* null mutant strain was dramatically higher than its WT control strain at the age of 5 generations (a representative generation for young cells). The 21 generation-old cells (a representative age for old cells) of the WT strain accumulated significant amount of protein-carbonyl, relative to its 5 generation-old cells (Fig. 1A). However, the protein-carbonyl level was still significantly lower than observed with the MT strain, respectively (Fig. 1A). To support the notion that the observed protein-carbonyl accumulations may be a consequence of enhanced oxidative stress, the protein-carbonyl accumulations in both yeast strains were monitored following exposure of the cells to H_2O_2. As expected, the MT strain accumulated 2.6-fold more protein-carbonyl relative to the WT strain under these conditions (Fig. 1B). It is important to note that the highest levels of accumulated protein-car-

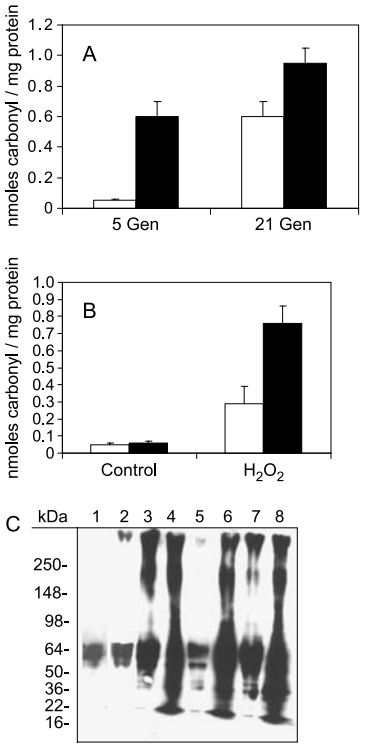

Fig. 1. Protein-carbonyl levels in *msrA* null mutant (MT) and wild-type control (WT) yeast strains. White bars represent WT yeast cells and Black Bars represent MT yeast cells. Each bar represents an average value resulting from three independent experiments. For both **A** and **B** the differences between WT and MT values were significant with $P < 0.001$ in all cases, except for the difference between WT and MT under control conditions in **B**. The protein-carbonyl measurements in **A** and **B** were carried out using a specrtrophotometric method following dinitrophenol absorbance at 360 nm. **A** Young yeast cells (5-generation old cells) and old yeast cells (21-generation old cells) were isolated by elutriation. The method for the isolation of young and old yeast mother cells depends on cell size as described by Laun et al. (2004), and under Materials and methods. *5 Gen* 5-generation old cells; *21 Gen* 21-generation old cells. **B** Yeast cells at logarithmic phase were inoculated into liquid YPD media in the presence or absence of 2 mM of H_2O_2 at 30 °C. When cell density reached early logarithmic phase ($A_{600} = 0.3$), the cells were spun down, washed three times with PBS, and disrupted. Following centrifugation, the resulting supernatants of the cell extracts were used for protein-carbonyl determination as described under Materials and methods. Control and H_2O_2 represent non-treated and H_2O_2-treated cells, respectively. *kDa* Molecular mass markers in kilo-Dalton. **C** Protein extracts of both yeast strains from **A** and **B** were probed for protein-carbonyl by western-blot analysis using antibodies against dinitrophenol (Cayman). *1–4* parallel the protein extracts used in **B**. *1* Wild type (WT) untreated control cells; *2 msrA* null mutant cells (MT) untreated control cells; *3* WT cells treated with H_2O_2 ; *4* MT cells treated with H_2O_2; *5–8* parallel the protein extracts used in **A**. *5* 5-generation old WT cells; *6* 5-generation old MT cells; *7* 21-generation old WT cells; *8* 21-generation old MT cells

bonyl, in both mother and H_2O_2-exposed cells, were similar (Fig. 1A, B).

The western blot analysis results, using anti-dinitrophenol antibodies (Fig. 1C), confirmed the pattern of protein-carbonylation of the WT versus the *msrA* null mutant strain as obtained by the specrtrophotometric measurements of protein bound-dinitrophenol (Fig. 1A, B). The reason that the untreated *msrA* null mutant cells (Fig. 1B) have far less accumulation of protein-carbonyl relative to its respective 5 generation-old mother cells (Fig. 1A) is suggested to be the following: the untreated *msrA* null mutant cells were less than 1 day old (early logarithmic phase) and comprised of both mother and daughter cells. However, the 5 generation-old cells comprised of only mother cells that were grown into mid- logarithmic phase. Obviously, the cells of the latter culture were more exposed to naturally occurring reactive oxygen species compare to the untreated cells, and consequently have accumulated more protein-carbonyl. In contrast, the WT strain was more protected from oxidative stress due to the presence of MsrA. This was further demonstrated by maintaining a low protein-carbonyl level at the 5 generation-old culture (Fig. 1A) that was compatible with the level shown in the untreated cells, respectively (Fig. 1B).

Accumulation of protein-carbonyl in human tissues (Moskovitz et al., 2002b) and in yeast mother cells (Aguilaniu et al., 2003) was shown to be one of the characteristics of aging (Moskovitz et al., 2002b). Based on the latter observations and the current presented results (Fig. 1), it is suggested that the MT strain is aging at a faster pace when compared to the WT strain. Additionally, lower antioxidant defense is associated with the progression of the aging process. Among the functions of the Msr system is to serve as antioxidant via its MetO-dependent reducing activities. Moreover, the MsrA activity was shown to decline in various mammalian tissues with age or oxidative stress (Moskovitz et al., 2001; Stadtman et al., 2002). Therefore, it was important to monitor the Msr activity as a function of age in both the *msrA* null MT and it control WT yeast strains.

As shown in Fig. 2, both the WT and the MT strains demonstrated a significant loss of total Msr activity at the 21 versus 5 generation-old cells. The observed decline in Msr activity with age in both examined yeast strains is similar to the activity reduction shown in most aged mammalian tissues (Stadtman et al., 2002). Also, the level of total Msr activity in the MT strain represents MsrB activity only, and its further decline at 21 generation-old cells may contribute to the additional protein-carbonyl accumulation at that age (Fig. 1A). However, lack of MsrA activity in the MT strain seems to have a major contribution to its protein-carbonyl accumulation at the 5 generation-old cells (Fig. 1A). The above results provide supportive evidence for the importance of the Msrs' activities in

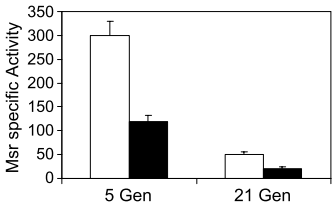

Fig. 2. Total Msr activity in *msrA* null mutant (MT) and wild type control (WT) yeast strains. Young cells (5-generation old cells) and old cells (21-generation old cells) were analyzed for total Msr activity using Dabsyl-MetO as substrate and DTT as a reduction power, according to the procedure described under Materials and methods. Msr specific activity is calculated as pmoles dabsyl-Methionine formed per mg protein per min. White bars represent WT yeast cells and black bars represent MT yeast cells. Each bar represents an average value resulting from three independent experiments. *5 Gen* 5-generation-old cells; *21 Gen* 21-generation old cells. For 5 Gen, $P < 0.001$; for 21 Gen, $P < 0.05$

preventing age-associated damage to proteins. Irreversible post-translation modification to proteins may result in their malfunction, aggregation, and thereby causing cellular damage leading to senescence and apoptosis. Consequently, removal of faulty proteins that accumulate as a function of age or oxidative stress conditions is essential for cell survival.

In summary, the present study reinforces the importance of the Msr system in protecting against age-dependent accumulative protein oxidation with age. Future research will be focused on determining the identity of the oxidized proteins in the *msrA* null mutant yeast strain and elucidating their role in the aging process.

Acknowledgment

This work was supported by the School of Pharmacy at the University of Kansas start-up funds.

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Verleger: Springer-Verlag GmbH, Sachsenplatz 4–6, 1201 Wien, Austria – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, 1090 Wien, Austria – Redaktion: Sachsenplatz 4–6, 1201 Wien, Austria – Hersteller: Satz und Umbruch: Thomson Press (India) Ltd., Chennai; Offsetdruck: Krips bv, Kaapweg 6, 7944 HV Meppel, The Netherlands – Verlagsort: Wien – Herstellungsort: Meppel – Printed in The Netherlands.