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The peptide methionine sulfoxide reductases, MsrA and MsrB (hCBS-1), are downregulated during replicative senescence of human WI-38 fibroblasts

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Abstract In contrast to other oxidative modifications of amino acids, methionine sulfoxide can be enzymatically reduced back to methionine in proteins by the peptide methionine sulfoxide reductase system, composed of MsrA and MsrB. The expression of MsrA and one member of the MsrB family, hCBS-1, was analyzed during replicative senescence of WI-38 human fibroblasts. Gene expression decreased for both enzymes in senescent cells compared to young cells, and this decline was associated with an alteration in catalytic activity and the accumulation of oxidized proteins during senescence. These results suggest that downregulation of MsrA and hCBS-1 can alter the ability of senescent cells to cope with oxidative stress, hence contributing to the age-related accumulation of oxidative damage.

Key words: Senescence; Protein oxidation; Methionine sulfoxide; Protein repair; Oxidative stress

1. Introduction

Normal human fibroblasts that undergo replicative senescence can be used as a model for studying human aging [1]. The processes which lead to replicative senescence, as well as to normal aging, are not yet well identified. One of the potential causal factors is the accumulation of reactive oxygen species (ROS)-mediated oxidative modification of cellular macromolecules, such as damaged DNA [2] and oxidized proteins. These damaged proteins are less active or inactive and are known to be deleterious to cellular function and homeostasis [3]. Moreover, the protein carbonyl content, which is an indicator of protein oxidation, increases significantly during aging in different cellular and physiological systems [4]. Most oxidative modifications of proteins are not reversible, and altered proteins are eliminated by the proteasomal system which degrades cytosolic oxidized proteins (reviewed in [5,6]). However, in contrast to other oxidation products of amino acids, methionine sulfoxide (Met(O)) can be enzymatically reduced back to methionine by an enzyme initially referred to as peptide methionine sulfoxide reductase (PMSR) [7]. Oxidation of methionine among a large number of proteins results in modification of their activities [8,9] and/or their conformational structure [10]. The original PMSR has been renamed MsrA, and has been shown to be specific to the *S*-enantiomer of Met(O) [11]. Recently, enzymes that catalytically reduced the *R*-enantiomer of Met(O) have been identified in several species and have been referred to as MsrB. Among them, hCBS-1 [12] was shown to act simultaneously with MsrA, leading to complete reduction of a protein such as calmodulin [13]. MsrA has been described as playing an important role in cellular defense against oxidative stress and in longevity in *Drosophila* and mouse [14,15].

In a previous study, we showed that MsrA gene expression decreases with age in rat organs, followed by a decrease in MsrA protein content and PMSR activity and an accumulation of oxidized proteins [16]. The purpose of the present work was to determine whether, during cellular senescence of human WI-38 fibroblasts monitored by proliferation rate and senescence-associated (SA) β-galactosidase activity, the status of MsrA and hCBS-1 is affected. Moreover, we tested modulation of expression of the MsrA and hCBS-1 genes after oxidative treatment of cells by H_2O_2 in order to determine their role in the ability of cells to adapt to oxidative challenges. Our results show that the SA downregulation of MsrA and hCBS-1 is accompanied by an accumulation of oxidized protein. Furthermore, MsrA and hCBS-1 are upregulated immediately after mild oxidative stress treatment, probably in order to allow cells to repair protein oxidative damage.

2. Materials and methods

2.1. Cell culture and senescence

All products for cell culture were purchased from Sigma. Human embryonic WI-38 cells were grown in Dulbecco's minimal essential medium (1 g/l glucose) supplemented with 10% fetal calf serum, 100

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Abbreviations: ROS, reactive oxygen species; PMSR, peptide methionine sulfoxide reductase; MsrA, peptide methionine sulfoxide reductase A; MsrB, peptide methionine sulfoxide reductase B; SA, senescence-associated; CPD, cumulative population doubling; Met(O), methionine sulfoxide

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U/ml penicillin, 100 μg/ml streptomycin and 20 mM L-glutamine. Cultures were kept in an incubator at 37°C up until 55 cumulative population doublings (CPD). Subconfluent cultures were obtained by seeding 6×10^4 cells/cm^2. WI-38 cells were classified into young fibroblasts corresponding to early-passage cells (below CPD 45), intermediate or middle-aged cells corresponding to intermediate-passage cells (CPD between 45 and 50), and old cells corresponding to late-passage cells (up to CPD 55). For H_2O_2 treatments, cells at 80% confluence were incubated in PBS buffer (1.30 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) containing various amounts of H_2O_2 (100–400 μM) for 30 min at 37°C. Control cells were incubated in PBS buffer during the same period. Activity assays and Western blot experiments were performed immediately after treatment.

2.2. SA β-galactosidase assays

Detection of SA β-galactosidase was performed in subconfluent cultures exhibiting 80% confluence, as previously described [17].

2.3. Determination of PMSR activity and Met(O) content in cellular homogenates

Cellular homogenates were obtained after disruption of cells by sonication in a buffer containing 10 mM HEPES, pH 8, 50 mM NaCl, 500 mM sucrose, 1 mM EDTA, 7.2 mM β-mercaptoethanol and 0.2% Triton X-100 at 4°C. Cell debris and organelles were removed from the crude extracts by centrifugation for 30 min at $20\,000\times g$ at 4°C. Protein concentrations were determined by the Bradford method using the protein microassay (Bio-Rad). PMSR enzymatic activity was determined in crude extract using *N*-acetyl[^{3}H]-Met-*R*,*S*(O) as substrate as previously described [18].

The protein-bound Met(O) level in cellular extracts was determined by the method previously described [19,20] with minor modifications. Briefly, 200 μg of cellular proteins were dialyzed against water for 3 h at 4°C and subjected to 100 mM CNBr. After lyophilization, the dried hydrolysates were dissolved in water and subjected to HCl hydrolysis for 48 h at 110°C in reducing conditions using the Pico-Tag system (Waters). The freeze-dried final mixture was then dissolved in water, derivatized by incubation with dinitrofluorobenzene for 15 min at 70°C and applied on a C18 column (Lichrospher 100 RP18) using a Beckman Gold HPLC system. Peaks corresponding to the different amino acids were detected at 360 nm and integrated.

2.4. Gel electrophoresis and Western blot experiments

Immunoblot experiments using anti-MsrA polyclonal antibodies [16] were performed after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) separation of 30 μg of WI-38 cellular proteins followed by electrotransfer onto a Hybond nitrocellulose membrane (Amersham-Biosciences). Immunoblot detection of carbonyl groups was performed with the OxyBlot oxidized protein detection kit (Chemicon International), according to the manufacturer. Densitometric analyses of the autoradiographies were performed using Image Master 1D software (Amersham-Biosciences).

2.5. RNA preparation and quantitative real-time polymerase chain reaction (PCR)

RNA extractions were performed using NucleoSpin RNA II (Macherey Nagel). A total of 2 μg of RNA was retrotranscribed for 1 h at 42°C by Moloney murine leukemia virus reverse transcriptase. Real-time PCR experiments were performed using SYBR Green as the intercalating agent (Roche Diagnostics). The specificity of PCR amplification products was checked by performing dissociation melting curve analysis and electrophoresis of PCR products (data not shown). Conditions of PCR were: 8 min at 95°C followed by 40 cycles of 10 s at 95°C, 10 s at 65°C and 10 s at 72°C. Quantification analyses of MsrA and hCBS-1 mRNA were normalized using the S26 ribosomal protein coding gene or the 18S rRNA as reference. In that case, PCR experiments were assayed with the Taqman PDAR 18S ribosomal RNA control probe (Reagent VIC™ probe, Applied Biosystems), and conditions of PCR were: 8 min at 95°C followed by 30 cycles of 10 s at 95°C and 40 s at 65°C. All experiments were done on a Roche Light Cycler (Roche Diagnostics). The sequences of the primers used for MsrA were 5′-TGGTTTTGCAGGAGGCTATAC-3′ and 5′-GTAGATGGCCGAGCGGTACT-3′ for sense and antisense respectively; the sequences of the hCBS-1 primers were 5′-CCGGAGCAGTTCTACGTCAC-3′ and 5′-TGAGCTTCACACTGCTTGCA-3′ for sense and antisense respectively.

2.6. Immunofluorescence labeling

Immunofluorescence labeling of MsrA in early- and late-passage WI-38 cells was carried out according to standard procedures. MsrA was localized by indirect immunofluorescence using a rabbit polyclonal anti-MsrA antibody diluted to 1/1000 in PBS buffer containing 2% bovine serum albumin and then an anti-rabbit IgG/fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma) diluted to 1/200 in the same buffer. For staining the mitochondria, a mouse antibody against human mitochondria (Chemicon International) was used at a dilution of 1/20 and was detected with a rhodamine-conjugated donkey anti-mouse secondary antibody (Chemicon International) diluted to 1/50. Incubations with primary and secondary antibodies were carried out at room temperature for 1 h each. After mounting, slides were observed using an Optiphoto2-Nikon epifluorescence microscope. The microscopy images were acquired with a Hamamatsu camera and then treated with Adobe Photoshop 6.0 Software.

2.7. Statistical analysis

Results, expressed as mean ± S.E.M., were compared by analysis of variance followed by Student's *t*-test for unpaired data. A value of $P<0.05$ was considered statistically significant.

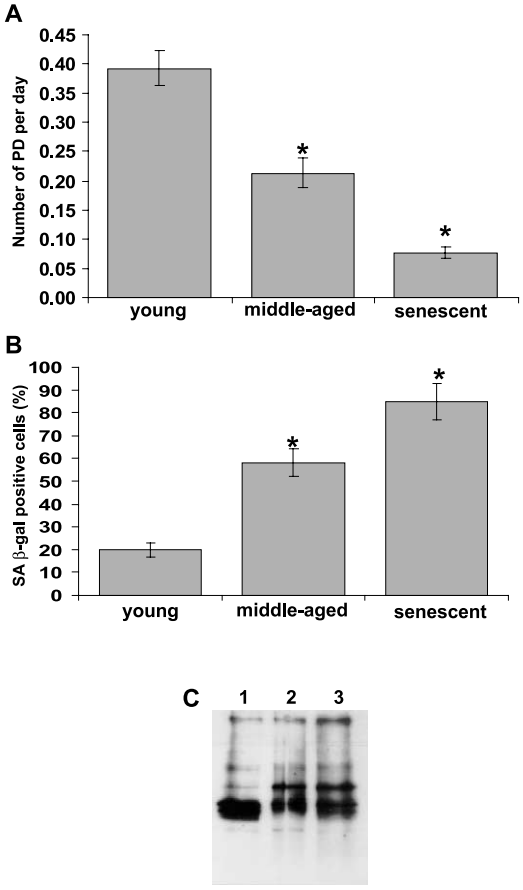

Fig. 1. Characterization of WI-38 human fibroblast cell cultures. CPDs were calculated at each passage using the formula: $PD = 3.32\times\log(Nc-Ns)$, where Nc is the number of cells counted at confluence and Ns the number of seeded cells. A: Decrease in replicative potential during serial passaging. B: Increase in SA β-galactosidase activity in WI-38 senescent cells. Data represent means ± S.E.M. from five experiments (*$P<0.05$ versus young cells). C: Accumulation of oxidatively modified proteins in middle-aged (lane 2) and senescent cells (lane 3), compared with young cells (lane 1).

3. Results

3.1. Replicative senescence of WI-38 fibroblasts is accompanied by an accumulation of protein oxidative damage

Serial passaging was continued until the cells permanently stopped dividing. The CPD level was calculated at each passage. Senescence of the WI-38 human fibroblasts used in these experiments was evaluated by the morphologic aspect of the cells as well as a decrease in replicative potential [21] and SA β-galactosidase activity detected at pH 6 [17]. Old cells presented a marked decrease in replicative potential (less than 0.1 population doubling per day for old cells) (Fig. 1A). In addition, β-galactosidase activity was observed in a high proportion of senescent cells (more than 80%), whereas young cells presented a low percentage of positive cells (less than 20%) (Fig. 1B). It is now well documented that aging is associated with an accumulation of macromolecule oxidative damage, especially protein oxidative modifications. All amino acids are targets of oxidation and some of them are converted to carbonyl derivatives [22]. To investigate the status of oxidatively modified proteins in WI-38 fibroblasts during serial passages, we monitored protein carbonyl content in cellular homogenates. As shown in Fig. 1C, an increase in high molecular weight modified proteins was observed in intermediate and late passages as compared with the earlier passages corresponding to young cells. The protein-bound Met(O) level was assessed in young and old cells as described in Section 2. In both cases, about 10% of Met residues were present as the Met(O) derivatives indicating that in contrast to the protein carbonyl content, no change in protein-bound methionine oxidation status could be observed during replicative senescence.

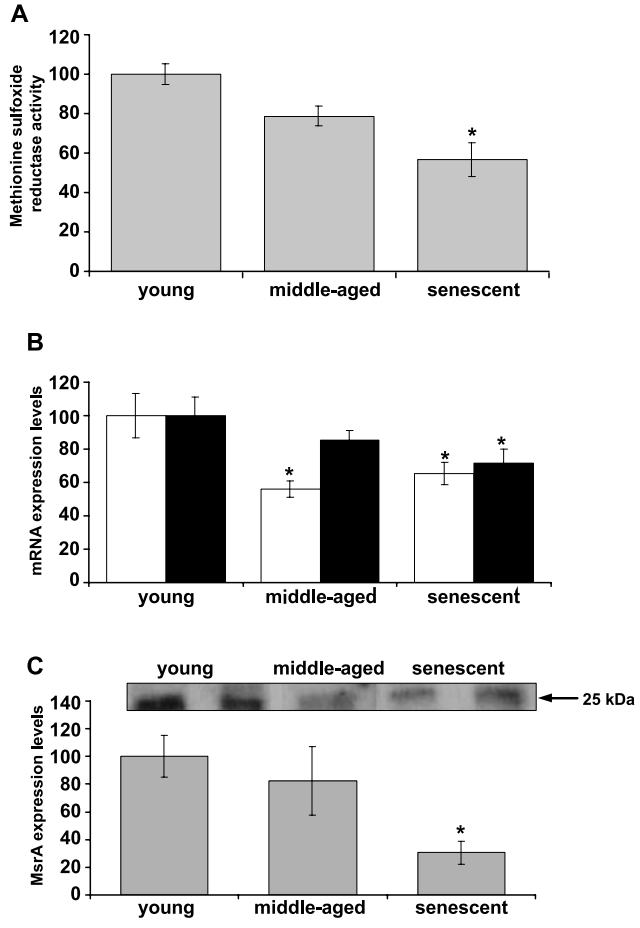

Fig. 2. Effect of cellular aging of WI-38 fibroblasts on Msr activity and expression levels of MsrA and hCBS-1. A: Activity of Msr was measured using *N*-acetyl[^{3}H]Met-*R*,*S*(O) as substrate in the presence of 100 mg of cellular proteins. The specific activity was 5.7 pmol of *N*-acetyl[^{3}H]Met/min/mg of protein for the early passage. This value was set at 100%. Values are means ± S.E.M. from three independent experiments (*$P < 0.05$ versus young cells). B: The levels of MsrA (white bars) and hCBS-1 (black bars) mRNA were evaluated by quantitative RT-PCR using a light cycler system and standardized with the housekeeping S26 protein or 18S rRNA gene. Results are presented as percent of transcript levels measured in cells from early passages. Values represent means ± S.E.M. of five independent experiments (*$P < 0.05$). C: Protein extracts were subjected to SDS–PAGE on 12% polyacrylamide, electrotransferred and immunoblotted with anti-MsrA antibodies. A typical blot from five independent experiments is shown. For quantification, the measurement corresponding to that obtained from young cells was taken as 100%.

3.2. Replicative senescence is associated with a decrease in MsrA and hCBS-1 expression

The SA accumulation of oxidized proteins may be due to a decline of oxidized protein elimination or to an increase in cellular ROS amounts, or both. In order to determine the status of PMSRs, we first examined whether the PMSR activity was affected during replicative senescence. We monitored the reduction of the synthetic substrate *N*-acetyl[^{3}H]Met-*R*,*S*(O) to *N*-acetyl[^{3}H]Met. As shown in Fig. 2A, a significant decline of 20 and 40% was observed in middle-aged cells and old cells, respectively, compared to their young counterparts. As a means to determine whether the senescence-related decline in PMSR activity was due to decreased enzyme expression, the mRNA levels of MsrA and hCBS-1 were determined in young, middle-aged and senescent cells using real-time RT-PCR. As shown in Fig. 2B, both of the mRNA levels decreased significantly. The decline was more significant for MsrA mRNA and the expression levels dropped to 40% ($P < 0.05$) as early as the pre-senescent stage. Furthermore, a significant 30% decrease ($P < 0.05$) was also observed when the hCBS-1 expression level was monitored in late-passage cells as compared to young cells. Next, the amount of MsrA protein was determined in cellular extracts prepared at different passages by Western blotting using anti-rat MsrA polyclonal antibodies. Overall protein expression observed in the different cell populations was in agreement with mRNA expression levels. In senescent cells, the amount of MsrA protein decreased significantly by 75% ($P < 0.05$) as compared to the young cells, whereas the difference observed between young and middle-aged cells was not significant (Fig. 2C).

3.3. Immunofluorescence analysis of MsrA in young and old cells

To determine whether quantitative differences in MsrA activity and content were associated with a different cellular distribution of the protein during senescence, immunofluorescence analysis of MsrA was performed in young and in senescent cells. As shown in the fluorescence microscopy images in Fig. 3, MsrA exhibited a strong grain-like signal compatible with mitochondrial localization (Fig. 3C, colocalization), and a diffuse signal in cytoplasm indicating that the protein was present in both cellular compartments, i.e. cytosolic and mitochondrial, as previously described [23,24]. The intracellular distribution pattern of MsrA seemed to be unchanged during

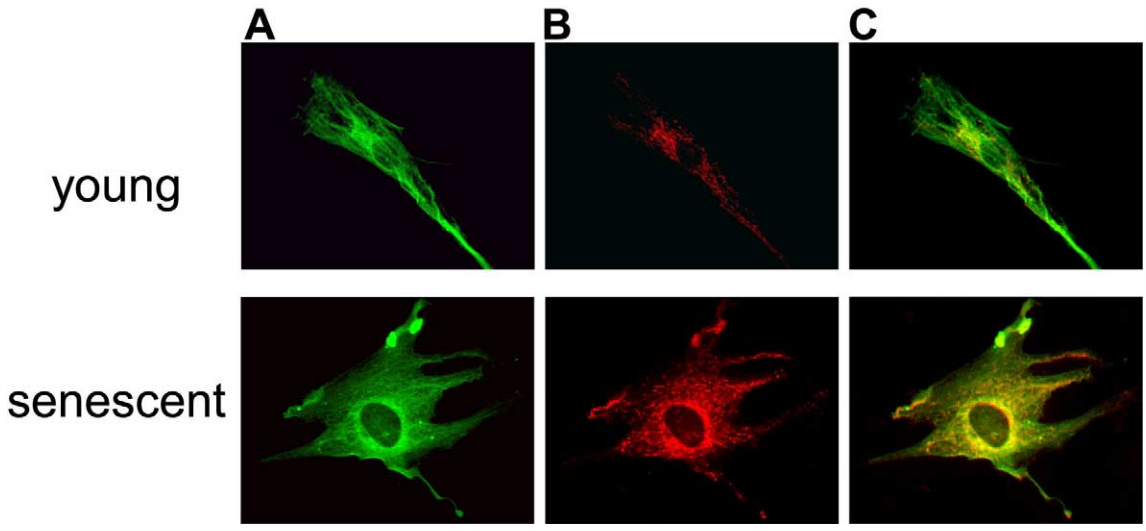

Fig. 3. Cellular distribution of MsrA by immunofluorescence microscopy. Cells from early and late passages were fixed, permeabilized and incubated with (A) primary antibody raised against MsrA and then with FITC-coupled secondary antibodies, or (B) primary antibody raised against mitochondria and rhodamine-coupled secondary antibodies. C: Overlays of MsrA and mitochondria-specific stains.

replicative senescence, although the extent of staining of structures that colocalized with mitochondria-specific staining increased substantially in old cells, especially in the perinuclear area. Since the mitochondrial mass was shown to increase during senescence [25], it was difficult to quantitatively compare the immunofluorescence signal in young and senescent cells.

3.4. *Stress-induced MsrA and hCBS-1 mRNA in WI-38 fibroblasts*

It has been shown that when cells are exposed for the first time to low concentrations of oxidants, they develop resistance to oxidative stress that can be regarded as oxidative stress adaptation. This resistance is accompanied by overexpression of several genes involved in antioxidant defense and damage removal and/or repair [26]. Since MsrA and hCBS-1 are considered to be regulators of antioxidant defense in mammals [14], we sought to investigate whether these enzymes are differentially modulated by oxidative stress in WI-38 cells. Total RNA was prepared from cells treated with various concentrations of H_2O_2 or control cells, and the steady-state level of MsrA and hCBS-1 mRNA was evaluated by real-time PCR. The transcript levels of MsrA and hCBS-1 increased significantly, by 70% ($P < 0.05$), when cells were treated with 100 μM of H_2O_2 (Fig. 4). For higher concentrations of H_2O_2, 200 and 400 μM, MsrA gene expression was the same as that of control cells. 50% overexpression of hCBS-1 was also observed when cells were treated with 100 or 200 μM of H_2O_2 and was abolished with 400 μM of H_2O_2.

4. Discussion

Replicative senescence has been described as a model of cellular aging. Indeed, although the mechanisms of cellular senescence are not completely known, it seems clear that such mechanisms are related to those controlling physiological aging. For example, ROS are known to be implicated in the aging process and in lifespan shortening in organisms [27] as well as in replicative senescence [28]. During this process, cells accumulate oxidized macromolecules, and especially oxidatively modified proteins. We and other investigators have previously shown that this accumulation can be explained at least in part by inhibition of the proteasomal system which is involved in the removal of oxidized proteins [29,30]. Moreover, among the few genes or proteins that are up- or downregulated during replicative senescence of human fibroblasts [31,32], the expression of certain proteasome subunits has been shown to decrease [29,33]. However, other maintenance systems, such as protein repair enzymes, might be altered in senescent cells. In the present study, we focused on the status of two PMSRs, MsrA and hCBS-1, during senescence of WI-38 fibroblasts. Our results indicated that gene expression decreased for both enzymes in senescent cells as compared to young cells, and that this decline was associated with alterations in PMSR activity and accumulation of oxidized proteins as demonstrated by protein carbonyl content measurement. In contrast, no modification in Met(O) level was detected during senescence. This result can be explained in the light of previous reports that have addressed the protein-bound Met(O) status in msrA knock-out yeast and mouse. In both models, no basal change in protein-bound

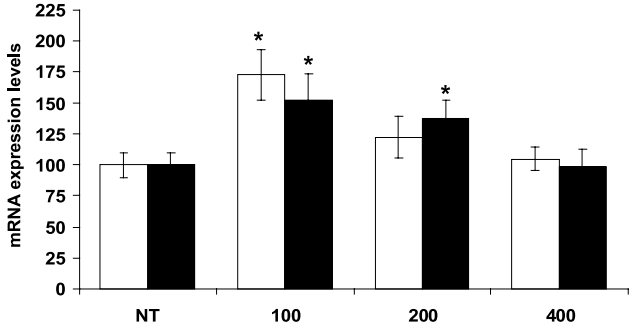

Fig. 4. Effect of H_2O_2 treatment on MsrA (white bars) and hCBS-1 (black bars) expression levels. The levels of MsrA and hCBS-1 gene expression were evaluated by quantitative RT-PCR using the light cycler system and standardized with the housekeeping S26 protein or 18S rRNA gene. Results are presented as percentage of the transcript levels measured in untreated cells. Values represent means ± S.E.M. of four independent experiments (*$P < 0.05$).

Met(O) level was detected for the mutant as compared to the wild type strain while a bigger increase was observed under oxidative stress conditions [34,35]. Indeed, the PMSRs were described not only as repair enzymes but also as antioxidant enzymes since, in proteins, the surface-exposed methionine residues can act as scavengers of a variety of oxidants. The catalytic reversion of Met(O) provides an amplification of the antioxidant potential of each methionine residue [36]. Taken together, these results suggest that downregulation of MsrA and hCBS-1 can alter the redox homeostasis of the cell, hence contributing to the accumulation of oxidative damage associated with senescence. Since MsrA has been described as an oxidized protein repair enzyme that may constitute an important antioxidant system [20], this enzyme has also been found in mitochondria, the major intracellular sources of ROS production [23,24]. According to these previous results, immunofluorescence experiments show that MsrA is detected in the two subcellular compartments of WI-38 fibroblasts, cytosolic and mitochondrial. MsrA seems to accumulate in mitochondria localized in the perinuclear area, probably to cope with the deleterious effects of overproduction of ROS that occurs in mitochondria during aging. A similar trend was observed for another antioxidant enzyme, glutathione *S*-transferase, that accumulated in the mitochondria of COS cells challenged with a lipid peroxidation product (4-hydroxynonenal), inducing intracellular oxidative stress [37].

The MsrA protein has been shown to be involved in antioxidant defense in several species. Although the antioxidant role of MsrB is less well documented, it is reasonable to postulate that this enzyme is also important in the cellular response to oxidative stress. Interestingly, when WI-38 fibroblasts were exposed to low concentrations of H_2O_2, a mild stress treatment, both Msr genes were upregulated, suggesting that these genes are necessary for cellular stress defense, probably enabling cells to repair their protein oxidative damage. Taken together, our results underscore the central role of the PMSRs not only in protein maintenance, but also in cellular defense and redox homeostasis.

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