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Original Research Communication

Overexpression of Methionine Sulfoxide Reductases A and B2 Protects MOLT-4 Cells Against Zinc-Induced Oxidative Stress

Filipe Cabreiro,[1] Cĕdric R. Picot,[1] Martine Perichon,[1] Bertrand Friguet,[1,2] and Isabelle Petropoulos[1,2]

Abstract

Among the amino acids, methionine is the most susceptible to oxidation, and methionine sulfoxide can be catalytically reduced within proteins by methionine sulfoxide reductase A (MsrA) and B (MsrB). As one of the very few repair systems for oxidized proteins, MsrA and MsrB enzymes play a major role in protein homeostasis during aging and have also been involved in cellular defenses against oxidative stress, by scavenging reactive oxygen species. To elucidate the role of zinc on the Msr system, the effects of zinc treatment on control and stably overexpressing MsrA and MsrB2 MOLT-4 leukemia cells have been analyzed. Here we show that zinc treatment has a pro-antioxidant effect in MOLT-4 cells by inducing the transcription of metallothioneins and positively modulating the activity of the Msr enzymes. In contrast, due to its pro-oxidant effect, zinc also led to increased cell death, reactive oxygen species production, and protein damage. Our results indicate that overexpression of the Msr enzymes, due to their antioxidant properties, counteracts the pro-oxidant effects of zinc treatment, which lead to a cellular protection against protein oxidative damage and cell death, by reducing the production of reactive oxygen species. *Antioxid. Redox Signal.* 11, 215–225.

Introduction

In cells, only a few amino acid oxidative modifications can be reversed back to the amino acid native form, such as the sulfur-containing amino acids, cysteine and methionine. Methionine residues are easily oxidized by all forms of reactive oxygen species (ROS) (9, 45), leading to the formation of two diasteroisomeric forms of methionine sulfoxide, methionine-S-sulfoxide (Met-S(O)) and methionine-R-sulfoxide (Met-R(O)). In proteins, the oxidized forms of methionine can be reduced catalytically by the action of the stereospecific enzymes MsrA and MsrB, respectively (14, 18, 25, 27, 51). In mammals, MsrA is expressed in almost all tissues with the exception of leukemia and lymphoma cell lines (25), and is localized in the cytosol (22, 26, 49), in the mitochondria (16, 26, 49), and in the nucleus (21, 22, 26). MsrB enzymes are highly expressed in cells of the immune system (1) and are present in all subcellular compartments. MsrB1 (SelX) is a selenoprotein present in the nucleus and the cytosol, MsrB2 (CBS-1) is found in the mitochondria, and MsrB3A/B, generated by alternative splicing, are targeted to the reticulum endoplasmic and to the mitochondria, respectively (20).

Msr overexpression studies have clearly demonstrated the implication of this oxidized protein repair system in the protection against oxidative stress. Overexpression of MsrA in human T-lymphocytes (35), PC-12 (52), lens (19), and WI-38 SV40 fibroblast cells (41), in yeast (35), or in whole organisms such as *Drosophila* (44) clearly increased their resistance against oxidative stress and protected the WI-38 SV40 fibroblasts against protein carbonyl accumulation (41). In addition, deletion of the msra gene in *E. coli* (37), yeast (34), mice (36), or in human lens cells (29) contributes for an increased sensitivity towards oxidative stress. In contrast, little is known about the role of MsrB; we have recently shown that overexpression of mitochondrial MsrB2 protects leukemia cells against oxidative stress-induced cell death and protein damage (5). In fact, methionine residues at the surface of pro-

[1]Laboratoire de Biologie et Biochimie Cellulaire du vieillissement, Université Paris-Diderot–Paris, Paris, France.
[2]Laboratoire de Physiologie et Physiopathologie, CNRS 7079 Université Pierre et Marie Curie–Paris, Paris, France.

teins have been proposed to serve as endogenous antioxidants scavengers, together with the catalytic action of methionine sulfoxide reductases (28) working as a ROS-detoxifying system. Studies have shown that overexpression or silencing of MsrA modulate the levels of cellular ROS (29, 41, 52), possibly through maintenance of mitochondrial function (29, 52).

All MsrBs are zinc-containing enzymes with zinc bound to the enzymes by the two cysteines present in two CxxC motifs that are characteristics of eukaryotic MsrBs. Zinc is believed to play a structural function in the core of the enzyme and mutation of one of four of those cysteines leads to the loss of the catalytic activity (24).

Zinc is an essential cofactor for many enzymes and is involved in many biological processes playing antagonist roles in the cells. Several studies suggest that redox-inert zinc has a ROS modulating pro-antioxidant role since it can enhance the cell's antioxidant capacity by maintaining an adequate level of metallothioneins (MTs) or through direct competition with other metals catalyzing the Fenton reaction, such as copper and iron (42). In contrast, other studies propose a pro-oxidant effect of zinc treatment provoking protein misfolding, promoting cell death and oxidative stress mainly through mitochondrial dysfunction (3, 10, 11, 17, 32).

Given its important implication in cellular processes, the concentration of zinc must be tightly controlled. Mammalian MTs are a class of nonenzymatic polypeptides of low molecular weight with high cysteine content responsible for cytosolic zinc buffering. Three forms of MT exist, the zinc fully loaded metallothionein (MT), the metal-depleted reduced form thionein (T_R) with an important reducing power, and the oxidized form thionin (T_O) (32). In fact, it has been proposed that alterations in the redox state of MTs are intrinsically linked to the regulation of zinc bioavailability and to other redox reactions (33). Several studies showing the induction of MTs by zinc (7,8) or upon oxidative stress conditions suggest an important antioxidant role for MTs (48), with a ROS scavenging ability similar to glutathione (46, 53).

We have recently shown that zinc supplementation in the elderly can help them to maintain plasma zinc homeostasis and to protect peripheral blood lymphocyte proteins against oxidative damage by positively modulating cellular protein maintenance systems such as the proteasome and the methionine sulfoxide reductases. In fact, we observed a 30% increase in the chymotrypsin-like activity levels of the proteasome and a 60% increase in Msr activity levels, accompanied by a 18% increase in MsrA protein levels (6). To investigate the role of zinc on the Msr system, we have analyzed the effects of zinc treatment on control and stably overexpressing MsrA and MsrB2 MOLT-4 human leukemia cells. Indeed, MOLT-4 cells are lacking MsrA and both MsrA and MsrB2 are localized in the mitochondria, the function of which is impaired by zinc treatment, hence resulting in ROS production. Here we show a dose-dependent dual response, namely pro-oxidant and pro-antioxidant, caused by the increasing levels of labile intracellular zinc on MOLT-4 cells. In addition, we show that overexpression of MsrA or MsrB2 can protect against the pro-oxidant damaging effects of zinc overload, namely increased protein oxidation, ROS production, and cell death observed in control cells. This protective threshold conferred by the overexpression of the Msr system allows the pro-antioxidant role of zinc, such as the upregulation of MTs and the increase in Msr activity, to be more effective in the protection of the MOLT-4 cells.

Materials and Methods

Transfection of MOLT-4 cell line by human MsrA and MsrB2 (hCbs-1) cDNA

Human MOLT-4 cell line (ATCC; CRL 1582) (LGC Promochem, Molsheim, France) were stably transfected with the pLXSN retroviral expression vector (BD Biosciences, Le Pont de Claix, France) based on the Moloney murine leukemia virus and Moloney murine sarcoma virus to generate a replication-deficient recombinant retrovirus containing the rat MsrA (39) and the human MsrB2 cDNA (I.M.A.G.E. clone, LGC Standards, Teddington, UK). The oligonucleotide primers flanking the open reading frame ($^{5'}$GCGAATTCATGCTCTCCGCCTCCAGAA$^{3'}$) and ($^{5'}$CGCGGATCCTTACTTTTTAATGGCCGTGG$^{3'}$), for MsrA and ($^{5'}$CGGAATTCATGGCGCGGCTCCTCTGGTT$^{3'}$) and ($^{5'}$GCAGATCTTCAGTGTTTCCTTGGTTTGAACTTCAAAGC$^{3'}$), for MsrB2, were used in a polymerase chain reaction, and the amplified DNA fragments were cloned into the *Eco*RI-*Bam*HI site of the pLXSN vector. The recombinant pLXSN vector was transfected using the liposome transfection reagent FuGene (Roche Applied Sciences, Meylan, France) into RetroPack PT67 packaging cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (Gibco® Invitrogen, Cergy Pontoise, France), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. To select stable transfectants, cells were grown in complete medium supplemented with 200 μg/ml Geneticin G418 antibiotics (Gibco® Invitrogen). The medium from the positive transfected cells was collected, filtered through a 0.45 μm filter, and diluted two-fold with fresh medium. The high-titer retroviral enriched medium was subsequently used to transduce the MOLT-4 cell line, cultivated in RPMI 1640 medium (Gibco® Invitrogen) supplemented with 10% (vol/vol) fetal calf serum, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C, 5% CO_2, and 95% humidity. Positive and stably transfected MOLT-4 cells were selected in RPMI 1640 complete medium with 200 μg/ml Geneticin G418 and tested for functional protein expression levels by immunoblot and Msr activity analysis.

Cell culture and zinc treatment

Stably transfected MOLT-4 cell lines with either pLXSN/MsrA, pLXSN/MsrB2 or pLXSN empty vector were propagated in 75 cm^3 plastic flasks (Greiner, VWR, Issy-les-Moulineaux, France) at 37°C, 5% CO_2, and 95% humidity, in complete RPMI 1640 medium containing 200 μg/ml Geneticin. For zinc treatment conditions, cells were seeded at a density of 1.3×10^6 cells/ml in fresh medium without fetal calf serum in 6- or 24-well plates. Cells were submitted to 24 h treatments with various concentrations of zinc sulfate ($ZnSO_4$) (ranging from 10 to 60 μM); proteins and RNA were then extracted as described below and used in further experiments. For the experiments using fluorescent probes, the cells were cultivated in RPMI 1640 medium without phenol red.

Cellular zinc content determination by fluorescence imaging

Intracellular zinc determination was assayed with the zinc-specific fluorogenic probe zinquin-ethyl-ester (Zinquin) (Sigma-Aldrich, Saint-Quentin Fallavier, France). Briefly, after treatment in nonsupplemented medium, cells were gently resuspended in 1 ml of pre-warmed PBS containing 25 μM final concentration zinquin probe and incubated at 37°C, 5% CO_2, for 30 min. As an autofluorescence control, PBS alone was tested. One aliquot of each sample was analyzed on a Nikon Eclipse TE2000-U microscopy (Nikon S.A.S, Champigny-sur-Marne, France) with excitation/emission wavelengths of 387 (± 11)/447 (± 60) nm. Fluorescence values correspond to the mean values obtained from 100 individual cells for each condition from three independent treatments and quantified by NIS-Elements software (Nikon S.A.S). For mitochondrial and Zinc co-labeling, cells were incubated at 37°C, 5% CO_2, for 45 min. in 1 ml of pre-warmed PBS containing 25 μM final of Zinquin probe and 100 nM of MitoTracker Red CMXRos probe (Gibco® Invitrogen). Mitochondrial labeling was analyzed with excitation/emission wavelengths of 531 (± 40)/593 (± 40) nm.

Cell viability assay

Cytotoxicity was determined by a colorimetric assay based on the cleavage of the yellow tetrazolium salt XTT (Sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells at a 1.4×10^5 cells/well density were harvested from the exponential-phase maintenance cultures and dispersed in 96-well plates. Formation of an orange formazan dye by metabolic active cells was measured using a micro-plate reader at 450 nm (Fluostar Galaxy, bMG, Stuttgart, Germany). The relative number of viable cells as compared to the nontreated cells was expressed as the percentage of proliferative cells.

Determination of Msr activity

Cellular homogenates were obtained from 20×10^6 cells using the CelLytic™ M mammalian cell lysis/extraction reagent (Sigma-Aldrich, Saint-Quentin Fallavier, France) at 4°C. Total Msr enzymatic activity was measured in cellular homogenates using *N*-acetyl-[^{3}H] Met *R*,*S* (O) as substrate, as previously described (2).

Light cycler RT-PCR analysis

The relative transcript levels of the Msr (msra, msrb1, msrb2, and msrb3A/B) and metallothionein genes (mt-1A, mt-1B, and mt-2A) were measured by semiquantitative RT-PCR. 2 μg total of RNA was reverse transcribed using SuperScriptTM III (Invitrogen) and the cDNA amplified by real-time PCR using the following specific primers for MsrA: forward 5′-TGGTTTTGCAGGAGGCTATAC-3′ and reverse 5′-GTAGATGGCCGAGCGGTACT-3′; MsrB1: forward 5′-GCGAGGTTTTCCAGAATCAC-3′ and reverse 5′-GGACACCTTCAAGGCTTCAG-3′; MsrB2: forward 5′-GCGACAGTCCACTCTTCAGTT-3′ and reverse: 5′-CCCAGGTCCATCAGGAAACA–3′; MsrB3A/B: forward 5′-CCGGGTCGTGTAGGGATAAA-3′ and reverse 5′-TGAGCACCACACTGAGAGCA-3′; MT1-A: forward 5′-TCCTGCAAATGCAAAGAGTG-3′ and reverse 5′-TTCCAAGTTTGTGCAGGTCA-3′; MT-1B forward: 5′-GAACTCCAGGCTTGTCTTGG-3′ and reverse 5′-GATGAGCCTTTGCAGACACA-3′; MT2-A: TCCTGCAAATGCAAAGAGTG and reverse 5′-CAGGTTTGTGGAAGTCGCGT-3′; 18S: forward 5′-ACATCCAAGGAAGGCAGCAG-3′ and reverse: 5′-TCGTCACTACCTCCCCGG-3′; β2-macroglobulin; forward: 5′-TTTCATCCATCCGACATTGA-3′ and reverse: 5′-CCTCCATGATGCTGCTTACA-3′. Transcripts were amplified for 35 PCR cycles with annealing temperatures of 60°C for all genes, with the exception of MsrB2 with an annealing temperature of 65°C. The reactions were performed using LightCycler® FastStart DNA Master^{Plus} SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) with efficiency >90%. The fluorescence values were normalized to those produced with primers for the 18S and β-2-macroglobulin housekeeping genes. The correlation coefficient between the two housekeeping genes was $R^2 = 0.9499$.

Subcellular fractionation, immunoblot, and oxyblot analyses

Subcellular fractionation was achieved using the mitochondria isolation kit for mammalian cells (Perbio Sciences, Brebieres, France) and performed according to the manufacturer's protocol. Alternatively, cellular homogenates were obtained using the celLytic™ M mammalian cell lysis extraction. MsrA, MsrB2, and MT-2A protein expression levels were determined by Western blot analyses. Total protein extracts were separated on 15% SDS–PAGE and electrotransferred. For the detection of MT-2A, protein extraction was done in the presence of 50 mM DTT. Western blotting experiments were performed with anti-MsrA antibody at a 1/20,000 dilution (39), with anti-MsrB2 at a 1/2,500 dilution (BioSynthesis, Lewisville, TX), antimetallothionein clone E9 (Dako, Trappes, France) at a 1/500 dilution, antimitochondrial aconitase (kindly provided by L. Szweda, OMRF, Oklahoma City, OK) at a 1/1,000 dilution, anti-20S proteasome (40) at a 1/8,000 dilution and anti-actin monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1/500 dilution used as loading control. Detection of carbonyl groups was performed with the OxyBlot oxidized protein detection kit (AbCys S.A., Paris, France) according to the manufacturer's protocol. 2.5 μg of total extract proteins were incubated for 15 min at room temperature with 2,4-dinitrophenylhydrazine to form the carbonyl derivative dinitrophenylhydrazone before SDS–PAGE separation. After transfer onto nitrocellulose, modified proteins were revealed by antidinitrophenol antibodies. Blots were developed with chemiluminescence using the SuperSignal West Pico chemiluminescent substrate (Perbio Sciences). Films were scanned and the amount of signal was quantified by densitometric analysis using ImageMaster 1D software (GE Healthcare Europe GmbH, France).

ROS measurements

ROS production was measured by the fluorescent probe dihydroethidium (DHE) (Molecular Probes®, Invitrogen). Msr overexpressing and control cells were incubated after 24 h $ZnSO_4$ treatment with 5 μM DHE for 45 min at 37°C, 5% CO_2. Analysis was performed on a FACScan flow cytometer

(Becton Dickinson, Le Pont-de-Claix, France) with a 488 nm excitation and a 610 nm emission filter.

Statistical analysis

All results are expressed as the mean ± SEM. Comparisons between control and Msr overexpressing cells under the different $ZnSO_4$ concentration treatments were analyzed with the Student *t* test and were assumed to be statistically significant if $p \leq 0.05$.

Results

Characterization of the MsrA and MsrB2 overexpressing MOLT-4 clones

MOLT-4 cells were transfected with the pLXSN empty vector or PLXSN/msra and pLXSN/msrb2 retroviral expression vector for stable transfection of MsrA and MsrB2, respectively. Positively transfected clones were selected with Geneticin, and the corresponding overexpressing clones were individually expanded. The levels of overexpression in both type of clones were checked at the protein level using anti-MsrA and anti-MsrB2 polyclonal antibodies and also at the activity levels that were measured using N-acetyl-[^{3}H]Met *R,S* (O) as substrate. Clones presenting higher levels of both expression and activity were selected for further analysis (data not shown). The transfection of msrA cDNA into MOLT-4 cells led to the presence of high levels of MsrA protein that was absent in the MOLT-4 control cells (Fig. 1A), in both the cytosolic and mitochondrial compartments (Fig. 1C), and consequently to a 20-fold increase in total Msr activity. Overexpression of MsrB2 in MOLT-4 cells is essentially restricted in the mitochondria (5) and leads to an increase of 3.5-fold in the protein expression levels accompanied by a 3-fold increase in the total Msr activity levels, compared to the control cells (Fig. 1B).

Increase of labile intracellular zinc is correlated with the increase of extracellular zinc concentration

To analyze the effects of extracellular added zinc on the levels of intracellular zinc in MOLT-4 cells, the behavior of labile zinc was visualized after 2 and 24 h of treatment using the fluorescent membrane-permeant probe zinquin. Visualization of control cells without added zinc revealed a low background fluorescence that did not change over time, indicating very low amounts of intracellular basal labile zinc that were not detected by this technique (Fig. 2A). Addition of 25 and 50 μM $ZnSO_4$ to the medium led to an increase of zinquin fluorescence, with a strong signal displayed in a punctuate manner, indicating that high amounts of zinc are limited to cytoplasmic vesicles. Indeed, when we labeled MOLT-4 mitochondria with MitoTracker in the presence of zinquin, we showed only a small overlap of Mitotracker and zinquin, indicating that zinc does not localize in mitochondria but in distinct cellular structures (Fig. 2C). It has been previously shown in HeLa cells that these cytoplasmic vesicles could be related to endosomes/lysosomes (7). No different pattern or distribution of zinc content was observed in the Msr overexpressing cells compared to the control cells (data not shown). Mean fluorescence quantified at 2 and 24 h clearly show that zinc continuously accumulates within treatment time (Fig. 2B). For 25 μM of zinc, there is a 2-fold increase in the intracellular concentration of labile zinc within treatment time, almost attaining the same concentration observed for 50 μM at 2 h of treatment. For 50 μM, the increase over time is much less pronounced than for 25 μM, suggesting an increase in the cellular zinc buffering capac-

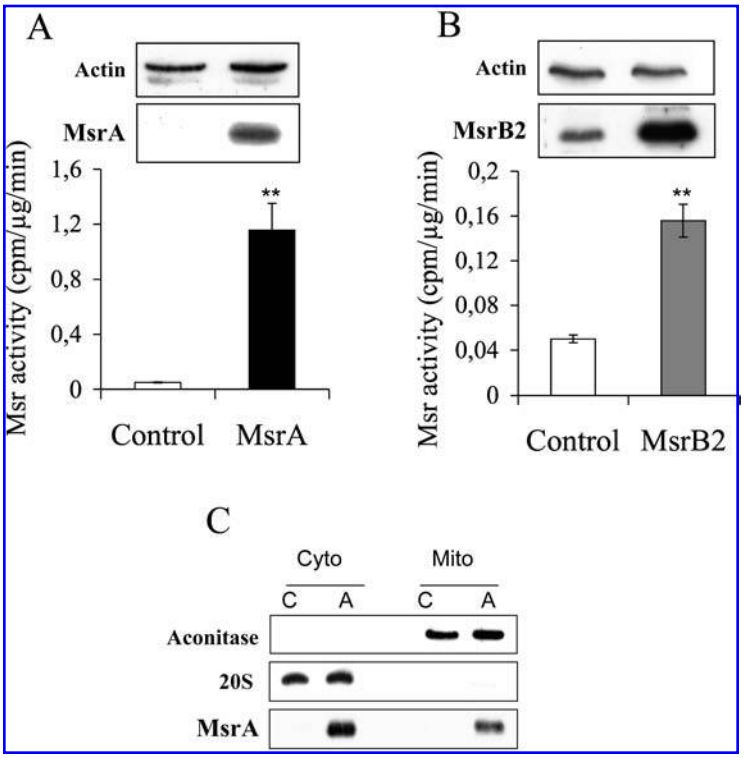

FIG. 1. Overexpression of MsrA and MsrB2 in MOLT-4 cells. Overexpression levels in MOLT-4 cells were checked by Western blot analysis and by measurement of the Msr activity using *N*-acetyl-[^{3}H] Met *R,S* (O) in total protein extracts (**A**) of MsrA overexpressing cells compared to control cells. (**B**) of MsrB2 overexpressing cells compared to control cells. β-actin was used as loading control. (**C**) Immunoblotting showing the relative protein level of MsrA in both control (C) and MsrA-overexpressing cells (A). Immunodetection of mitochondrial aconitase and 20 S proteasome were used as subcellular fractionation controls. The *bar graphs* show the increase in activity with respect to the vehicle controls and represent means ± SEM of at least three independent experiments. Values with statistical significance were obtained by Student's *t* test and considered for *p* under 0.05 (**$p < 0.01$).

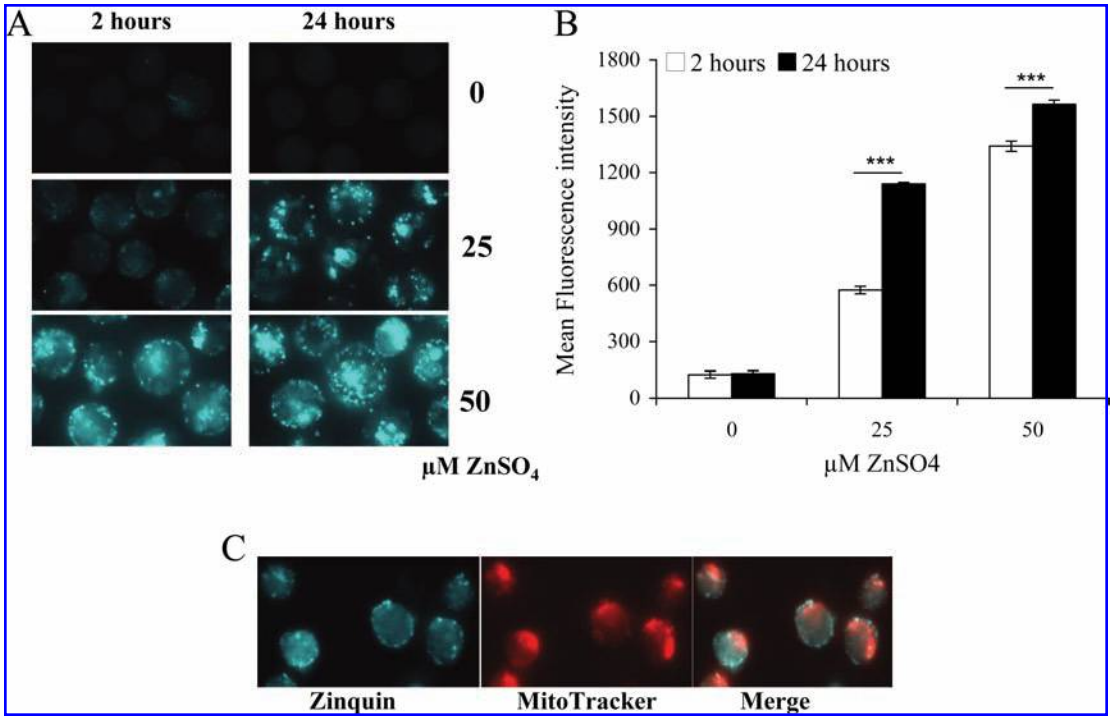

FIG. 2. Effect of added extracellular zinc on intracellular labile zinc concentration analyzed by zinquin probe in MOLT-4 cells. (**A**) Control cells were incubated with 25 and 50 μM $ZnSO_4$ concentrations for 2 and 24 h and analyzed by fluorescence imaging. (**B**) *Bar graphs* represent the mean intensity fluorescence values ± SEM obtained from 100 individual cells for each condition from three independent treatments. Values with statistical significance were obtained by Student's *t* test and considered for *p* under 0.05 (***$p < 0.001$). (**C**) Control cells were incubated with 50 μM $ZnSO_4$ concentration for 2 h and analyzed for zinquin and MitoTracker red fluorescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

ity. These results indicate that zinc added in the extracellular medium is responsible for an increase in the intracellular labile zinc concentration and that zinc continuously accumulates over treatment time.

Zinc treatment upregulates metallothionein but not methionine sulfoxide reductases gene and protein expression

To test the effect of zinc treatment on the transcription levels of metallothioneins and Msr enzymes, we checked the RNA levels of MT-1A, MT-1B, MT-2A, MsrA, MsrB1, MsrB2, and MsrB3 A/B. Real-time PCR experiments were performed on total RNA samples obtained from control cells treated with different zinc concentrations. All PCR reactions displayed efficiencies >90% and the results obtained were normalized with those obtained from two housekeeping genes (18S rRNA and β2-macroglobulin). Under the experimental conditions used, we could not detect any basal RNA expression of MT-1A, MT-1B, MsrA, and MsrB3 A/B. Instead, MT-2A, MsrB1, and MsrB2 RNA levels were easily detectable. Zinc treatment led to a 4-fold significant increase in the MT-2A transcription levels at 25 μM of added zinc and a massive 24-fold increase at 50 μM (Fig. 3A). On the contrary, zinc treatment had no effect on the RNA expression levels of both MsrB1 and MsrB2 at all concentrations used. In addition, in Msr overexpressing cells, as for control cells, there was a similar induction effect on MT-2A transcription levels but no effect of zinc treatment on the RNA levels of both MsrB1 and MsrB2 (data not shown).

A similar trend for zinc treatment was also observed at the protein expression levels (Fig. 3B). In fact, the increase in the RNA levels were accompanied by an increase in MT protein levels upon zinc treatment on both the control and Msr overexpressing cells. No changes in the protein expression levels were observed for the MsrA and MsrB2 protein in both control and Msr overexpressing cells.

Zinc treatment positively modulates endogenous methionine sulfoxide reductase activity

We have further analyzed whether intracellular zinc could modulate the total Msr activity (Fig. 4). After a 24 h treatment of $ZnSO_4$, we monitored the reduction of the synthetic substrate N-acetyl[^{3}H]Met *R,S* (O) in both control and Msr overexpressing cells. The results indicate a significant increase in the total Msr activities of control cells at all zinc concentrations used, compared to the nontreated counterparts. In contrast, the effect of increased intracellular concentration of zinc in MsrA and MsrB overexpressing cells is much less pronounced and only statistically significant for higher doses of zinc treatment. In parallel, total protein extracts obtained from nontreated control cells incubated in the presence of equal concentrations of zinc also showed a positive modulation of total Msr activities, although to a lesser extent of ~1.5-fold at 50 μM of zinc (data not shown).

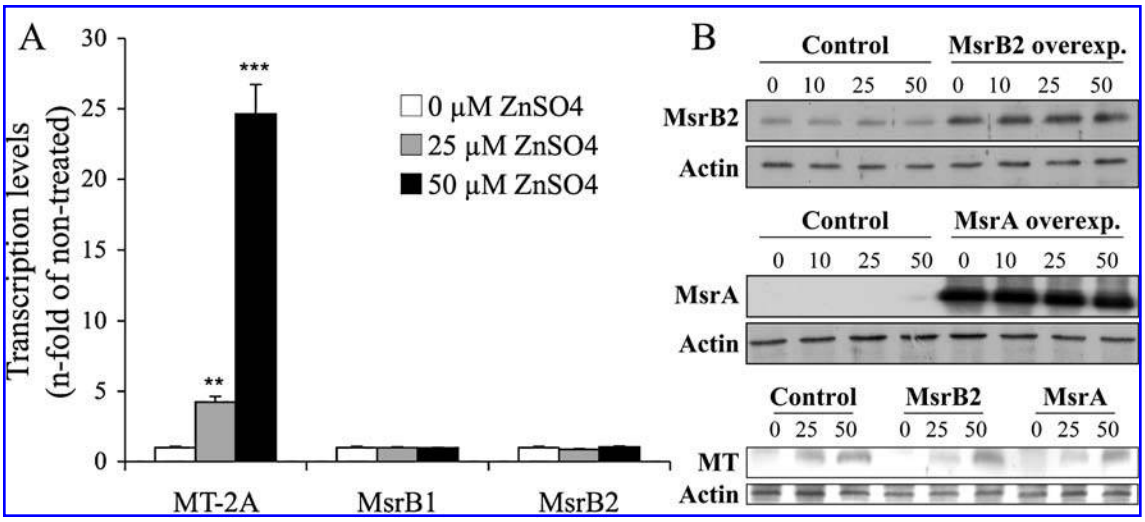

FIG. 3. Transcription and expression levels of metallothioneins are modulated upon zinc treatment but not those of the Msr members. Cells were treated with different doses of $ZnSO_4$ for 24 h. (**A**) 2 μg total RNA was reverse transcribed and the cDNA amplified by real-time PCR using specific primers for MT2-A, MsrB1, and MsrB2. The PCR values were normalized to those produced with primers for 18S and β2-macroglobulin. The graph shows changes in transcription levels with respect to untreated controls. The data represent means ± SEM of at least three independent experiments. Values with statistical significance were obtained by Student's *t* test and considered for *p* under 0.05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (**B**) Western blot analysis of control and Msr overexpressing cells treated with different doses of $ZnSO_4$ for 24 h using anti-MsrB2, anti-MsrA, and antimetallothionein antibodies. β-actin was used as loading control. The data represent means ± SEM of at least five independent experiments.

MsrA and MsrB2 overexpression protects MOLT-4 cells against zinc-induced cell death

Zinc has been previously shown to induce cell death in MOLT-4 cells (15). In order to address the protective role of MsrA and MsrB2 against cell death induced by zinc treatment, we treated both control and Msr overexpressing cells with different concentrations of $ZnSO_4$ (ranging from 0 to 50 μM) for 24 h. Cell survival was assayed by XTT test (Fig. 5). In control cells, the results indicate that zinc induces cell death in a dose-dependent manner. At the higher dose of $ZnSO_4$ applied, there is a 50% loss in viability of control cells. However, at all zinc concentrations used, there is a clearly protective effect by the overexpression of MsrA or MsrB2 with a major protection in the order of 30% for 50 μM of zinc treatment. In addition, for the Msr overexpressing cells, there was a low but significant positive effect on proliferation at the lowest zinc concentration used that is more pronounced for MsrB2 overexpressing cells (Fig. 5). Taken together, these data clearly show an overall protection against zinc-induced cell death in both MsrA and MsrB2 overexpressing cells when compared to control cells and even a positive effect on the proliferation at the lowest doses applied.

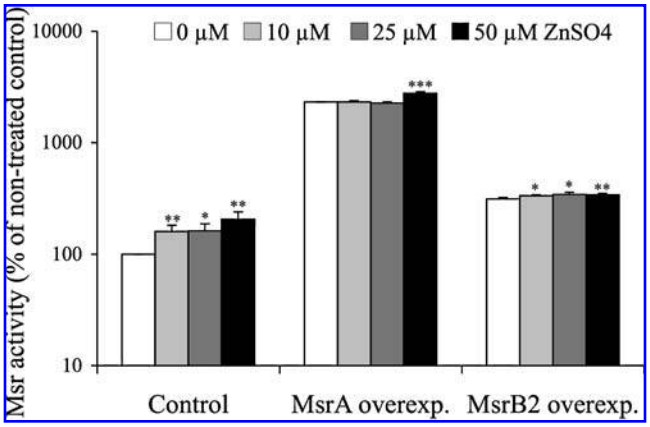

FIG. 4. Zinc treatment modulates Msr activity levels of control, MsrA, and MsrB2 overexpressing MOLT-4 cells. Control and overexpressing cells were treated with different doses of $ZnSO_4$ for 24 h and Msr activity was measured using *N*-acetyl-(^{3}H) methionine *R,S* sulfoxide as substrate in total protein extracts. The graph shows changes in Msr activity with respect to nontreated vehicle controls. Data were normalized using the nontreated vehicle control cells (specific activity = 0.05 ± 0.003 cpm/μg/min). The data represent means ± SEM of at least five independent experiments. Values with statistical significance were obtained by Student's *t* test and considered for *p* under 0.05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Overexpression of MsrA and MsrB2 decreases ROS production induced by zinc treatment and prevents accumulation of oxidatively modified proteins

It has been proposed that the Msr enzymes could serve as potential antioxidant enzymes through the reduction of exposed methionine sulfoxide in proteins and consequently, act as a ROS-scavenging system. Intracellular ROS production by the mitochondria provoked by zinc treatment was monitored using the fluorescent probe DHE that is mostly sensitive to superoxide ions. In order to evaluate whether Msr overexpression can affect the intracellular ROS production provoked by zinc, we measured the level of intracellular ROS after 24 h of treatment in both control and Msr overexpressing cells treated with different zinc concentrations (Fig. 6A). The results obtained show that zinc treatment in control cells leads to an increase in ROS production for the

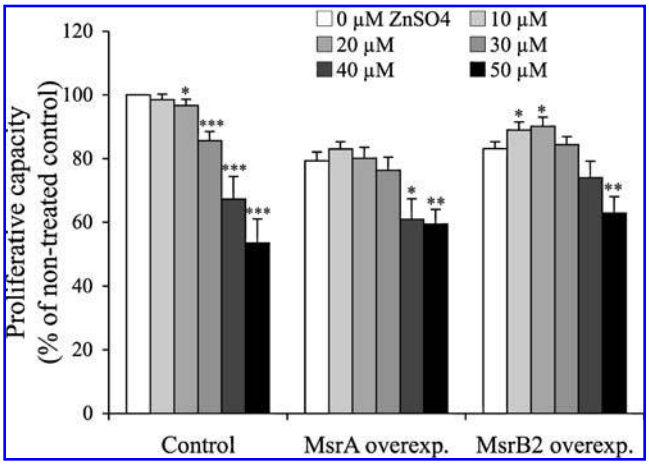

FIG. 5. Proliferative capacity of MsrA and MsrB2 overexpressing MOLT-4 cells is protected when compared to control cells upon zinc treatment. Control and overexpressing cells were treated with different doses of $ZnSO_4$ for 24 h, and cell proliferation was assessed by the Cell Proliferation XTT assay. Data were normalized using the nontreated vehicle control cell absorbance at 450 nm as 100%. The data represent means ± SEM of at least five independent experiments. Values with statistical significance were obtained by Student's *t* test and considered for *p* under 0.05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

two concentrations used, with an increase of ~60% at the highest dose applied (50 μM $ZnSO_4$). In contrast, at 25 μM of $ZnSO_4$, both Msr overexpressing cells show a slight decrease in ROS production below the basal level. At the higher dose, an increase in ROS production is observed but still presenting a 30% decrease when compared to the level measured in control cells. These results point out the fact that the overexpression of Msr enzymes provides an antioxidant buffering capacity capable of counteracting the production of ROS caused by zinc treatment.

To investigate the status of oxidatively modified proteins in control and Msr overexpressing cells upon zinc treatment, we monitored protein carbonyl content in cellular homogenates using Oxyblot (Fig. 6B). Similar levels of protein carbonyls were observed at the basal level amongst the control and the Msr overexpressing cells. Relative quantification of total carbonyl derivatives indicated that the carbonyl content increased upon zinc treatment for 24 h in control cells in a dose response manner (Fig. 6C). In contrast, zinc treatment in MsrA and MsrB2 overexpressing cells had the opposite positive effect, leading to a reduction in the protein carbonyl levels, with a lower carbonyl content at the most important dose of zinc (50 μM $ZnSO_4$). These results indicate that zinc treatment in MOLT-4 cells contributes for an increase on the protein carbonyl content while in Msr overexpressing cells, oxidative protein damage is reduced upon treatment most likely due to the decreased ROS production and increased protein maintenance potential of these cells.

Discussion

The Msr system represents an important intracellular oxidized protein repair and antioxidant system and is composed of MsrA and MsrB that catalyze the reversion of the Met-S(O) and Met-R(O), respectively, to the reduced form of methionine within proteins (50). In this article, we have analyzed the role of zinc on the Msr system in MsrA and MsrB2 overexpressing cells, compared to human leukemia MOLT-4 control cells. Indeed, only zinc-containing MsrB enzymes (namely MsrB1 and MsrB2) are present in MOLT-4 cells. We have found that zinc may have a deleterious pro-oxidant effect on MOLT-4 cells and that overexpression of MsrA or MsrB2 was able to counteract this effect and to potentate the pro-antioxidant effect of zinc in MOLT-4 cells. Interestingly, both MsrA and MsrB2 are localized in the mitochondria that are believed to be one of the targets for zinc pro-oxidant damaging effects. In addition, it has been previously shown that overexpression of MsrA in PC12 cells protects mitochondria against hypoxia/reoxygenation-induced oxidative damage (53) while overexpression of MsrB2 in MOLT-4 protects mitochondria against H_2O_2-induced oxidative stress (5).

We have previously shown that a dietary complementation of zinc in elderly increased the Msr activity in peripheral blood lymphocytes, thus enhancing the cellular antioxidant defences (6). On the contrary, zinc deficiency in CD-1 mice seems to have no visible effect on Msr in liver and kidney (47). Zinc treatment of control and Msr overexpressing MOLT-4 cells leads to a rise in intracellular free zinc and to an increase in Msr activity with no corresponding modifications of RNA and protein levels of any of the known endogenous Msr members (MsrB1 and MsrB2) nor of the overexpressed MsrA and MsrB2. Zinc could enhance the Msr activity through the gain of an essential cofactor which could be zinc itself, since MsrB enzymes are dependent of zinc binding. In fact, when MOLT-4 control cells protein extracts were incubated with 50 μM of ZnSO4, a 1.5-fold increase in Msr activity was observed. Another explanation could be the upregulation by zinc of an inducible unknown Msr member, probably exhibiting Met-S(O) reductase activity, since MOLT-4 cells are among the few cell lines that do not contain endogenous MsrA. This increase in endogenous Msr activity is not sufficient to maintain an adequate cellular redox homeostasis in these cells, as shown by the build-up of oxidized proteins and the increase of ROS production, mostly superoxide, by using the DHE fluorescent probe. However, several studies have shown that superoxide detection by DHE remains questionable since the specificity of DHE for superoxide *in vivo* is limited by autooxidation as well as by nonsuperoxide-dependent cellular processes that can contribute to the oxidation of DHE (54). It has also been recently suggested that superoxide detection upon excitation at 510 nm was usually overestimated (43). Nevertheless, DHE fluorescence measurements remains widely used for the detection of superoxide in cultured cells. In fact, it is known that zinc overload can trigger intracellular ROS production through mitochondrial and nonmitochondrial pathways. Zinc, by inactivation of dihydrolipoamide dehydrogenase consequently preventing NADH production and by inhibition of the complex III of the respiratory chain, contributes to mitochondrial dysfunction (12). On the other hand, it can increase the activity of a NADPH oxidase though protein kinase C activation (38), leading to an increased production of intracellular ROS. It has also been proposed that alterations in the redox state of MTs, which are considered as an im-

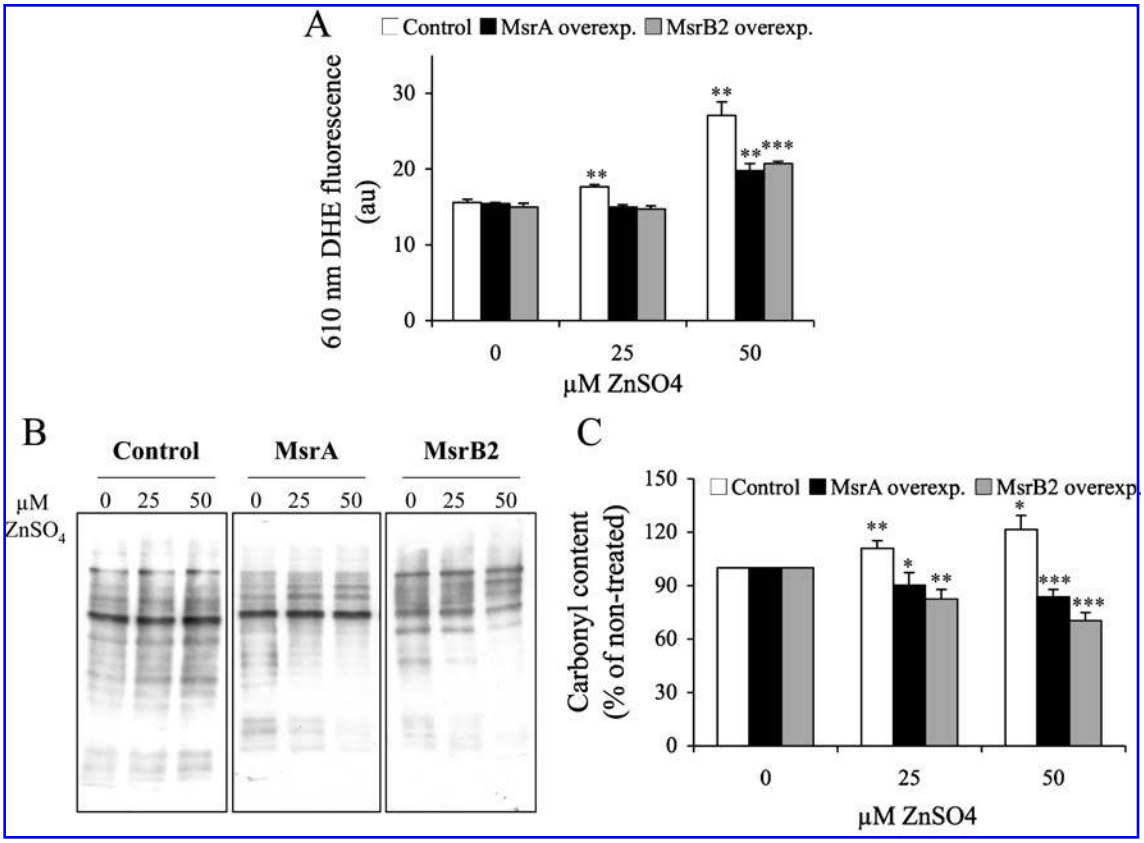

FIG. 6. Overexpression of MsrA and MsrB2 decreases superoxide production induced by zinc treatment and protects against protein oxidative damage. (**A**) Representative bar graph showing the generation of ROS using DHE assessed by flow cytometry, in both control and Msr overexpressing cells treated with different concentrations of $ZnSO_4$ for 24 h. The data represent means ± SEM of at least three independent experiments (au: arbitrary unit). (**B**) Oxidized proteins were determined by detecting carbonyl content with OxyBlot in control and overexpressing cells upon a 24 h zinc treatment. (**C**) The graph shows changes in oxidized protein content with respect to nontreated controls of each cell type (internal control of each independent experiment). Quantification of the blots was performed using ImageMaster 1D software. The data represent means ± SEM of at least five independent experiments. Values with statistical significance were obtained by Student's *t*-test and considered for *p* under 0.05 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

portant antioxidant system (48), with a ROS scavenging ability similar to glutathione (46, 53), regulate zinc bioavailability and other redox reactions (31). Moreover, it has been recently shown that the formation of disulfide bonds with the cysteine residues of MTs and homocysteine, which is highly reactive and continuously formed in the ubiquitous methionine cycle, disrupts zinc binding by the protein (13). According to this principle, the release of readily available intracellular zinc from MTs in an oxidative stress condition, not only promotes additional ROS production, but also a consequent zinc release that will induce the cell into a potentially dangerous vicious cycle. However, the zinc-mediated increase in ROS observed in MOLT-4 cells can be counteracted, at least in part, by the overexpression of MsrA or MsrB2. Interestingly, MsrA overexpression in WI38-SV40 fibroblasts has been previously shown to upregulate the dihydrolipoamide dehydrogenase (4).

In mammals, overexpression of MsrA in human T-lymphocytes, PC-12, lens, and WI-38-SV40 fibroblast cells (19, 35, 41, 52) protect them against oxidative stress. In contrast, msra null mouse exhibit increased sensitivity to oxidative stress associated to a shortened lifespan (36). These antioxidant properties of the Msr system has been attributed to the cyclic reversible oxidation/reduction of the surface-exposed methionine residues in proteins that can act as scavengers of a variety of oxidants (28). This effect is less documented for MsrB2, but short interfering RNA mediated gene-silencing of each of the three MsrB in human lens cells resulted in increased oxidative-stress-induced cell death, suggesting an important role for MsrB in lens cell viability and oxidative stress protection (30). More recently, we have provided evidence that the overexpression of MsrB2 preserve mitochondrial integrity upon H_2O_2-induced oxidative stress by preventing the mitochondrial intracellular ROS build-up through its scavenging role and also lead to the protection of the stress-induced inactivation of the proteasome (5). Since MsrB2 is localized in mitochondria (23) and MsrA in both cytosol and mitochondria, whether the observed protection of MOLT-4 cells against zinc-induced oxidative stress could be mediated by direct scavenging of ROS, such as superoxide, or by repair of certain mitochondrial proteins remains to be elucidated. Increased oxidative stress is believed to affect proteins since amino acids are very sensitive to almost all forms of ROS. In fact, we have previously shown an ac-

cumulation of oxidatively modified proteins in WI38-SV40 submitted to H_2O_2 treatment (41). Similarly, zinc treatment of MOLT-4 cells results also in an increase of carbonylated proteins in the cell and overexpression of the repair Msr enzymes prevents the formation of irreversibly oxidized proteins. It is interesting to note that although the MsrA and the MsrB enzymes have unique and different stereospecific activity, overexpression of only one of these enzymes is sufficient to counteract the increase in cellular ROS and oxidized protein content upon zinc treatment. An identical protecting effect was observed with MsrA overexpressing cells that exhibit higher levels of Msr activity compared to MsrB overexpressing cells, indicating that this effect seems not to be dependent of the level of Msr activity. This could be explained by the presence of a limiting factor, such as methionine availability for oxidation or due to the regenerating system of the Msr catalytic process.

Taken together, our results provide new insights in the role of zinc on cellular redox regulation and indicate that zinc has a pro-oxidant effect on MOLT-4 cells perturbing their redox homeostasis by increasing the ROS production and the level of oxidative damages. The oxidized protein repair enzymes MsrA and MsrB2, when overexpressed in the cells, are able to counteract the zinc-mediated damaging effects. The interplay between zinc and the antioxidant properties of these enzymes should be further analyzed to understand how Msr proteins could potentiate the pro-antioxidant role of zinc.

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Abbreviations

DHE, dihydroethidium; DTT, dithiothreitol; Met-R(O), methionine-R-sulfoxide; Met-S(O), methionine-S-sulfoxide; MT, metallothionein; MsrA, methionine sulfoxide reductase A; MsrB, methionine sulfoxide reductase B; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Zinquin, zinquin-ethyl-ester; $ZnSO_4$, zinc sulfate.

Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Isabelle Petropoulos
Laboratoire de Biologie et Biochimie Cellulaire du Vieillissement
EA 3106 / IFR 117
Université Paris Diderot–Paris 7
2 place Jussieu, Tour 33-23, 1er étage, CC 7128
75251 Paris cedex 05, France

E-mail: isabelle.petropoulos@snv.jussieu.fr

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