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Prolonged selenium deficient diet in *MsrA* knockout mice causes enhanced oxidative modification to proteins and affects the levels of antioxidant enzymes in a tissue-specific manner

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Abstract
The methionine sulfoxide reductase (Msr) system (comprised of MsrA and MsrB) is responsible for reducing methionine sulfoxide (MetO) to methionine. One major form of MsrB is a selenoprotein. Following prolonged selenium deficient diet (SD), through F2 generation, the *MsrA* − /− mice exhibited higher protein–MetO and carbonyl levels relative to their wild-type (WT) control in most organs. More specifically, the SD diet caused alteration in the expression and/or activities of certain antioxidants as follows: lowering the specific activity of MsrB in the *MsrA* − /− cerebellum in comparison to WT mice; lowering the activities of glutathione peroxidase (Gpx) and thioredoxin reductase (Trr) especially in brains of *MsrA* − /− mice; elevation of the cellular levels of selenoprotein P (SelP) in most tissues of the *MsrA* − /− relative to WT. Unexpectedly, the expression and activity of glucose-6-phosphate dehydrogenase (G6PD) were mainly elevated in lungs and hearts of *MsrA* − /− mice. Moreover, the body weight of the *MsrA* − /− mice lagged behind the WT mice body weight up to 120 days of the SD diet. In summary, it is suggested that the lack of the *MsrA* gene in conjunction with prolonged SD diet causes decreased antioxidant capability and enhanced protein oxidation.

Keywords: *Oxidative stress, post-translation modification, aging, antioxidants, protein oxidation*

Abbreviations: *MsrA/B, methionine sulfoxide reductase A and B; MetO, methionine sulfoxide; Trr, thioredoxin reductase; SelP, selenoprotein P; GPx, gluthathione peroxidase; G6PD, glucose 6-phosphate dehydrogenase; SD, selenium deficient diet; DA, selenium adequate diet;* MsrA − /−*, methionine sulfoxide reductae knockout; WT, wild-type*

Introduction

Accumulative post-translational 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, oxidized methionine (methionine sulfoxide, MetO) in proteins can be reversed to methionine by the methionine sulfoxide reductase (Msr) system (consists of MsrA which reduces *S*-MetO and MsrB which reduces *R*-MetO, thioredoxin reductase (Trr), thioredoxin (Trx) and NADPH) [1,2]. 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 hypersensitivity to oxidative stress and shorter life span, as shown in several organisms lacking the MsrA protein [3,4]. A major biological role of the Msr system is suggested by the fact that the *MsrA* null mouse (*MsrA* − /−) is more sensitive to oxidative stress, accumulates higher levels of carbonylated protein and

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has a shorter life span (by ~40%) than wild-type (WT) mice [5]. Furthermore, overexpression of MsrA in human T cells, plant and flies protects them from oxidative stress toxicity and leads to an almost doubling of the life span of flies [6–8]. In addition, selenium (Se) and MsrA are positive expression-regulators of the selenoprotein form of MsrB and Trr [5,9]. Consequently, weaned pups of first mouse generation (F1), fed with selenium deficient (SD) diet, exhibited higher protein–MetO and carbonyl levels detected as early as 6 months of age [9]. The protein–MetO and carbonyl levels observed in tissues of young mature mice fed with SD diet, resembles the levels seen in middleaged mice (13 months of age) fed with selenium adequate (SA) diet [9]. Accordingly, it was concluded that Se deficiency shortens the time required to cause significant accumulation of faulty proteins due to age-related oxidative stress. Previously, we have shown that exposing mice to 100% oxygen (hyperoxia) caused higher elevation of protein–carbonyl [5]. However, exposing mice to SD diet probably better mimics physiological condition of oxidation; thereby enabling us to follow the appearance of post-translational protein-modification (such as protein–MetO) especially in the *MsrA* mice. The current study examines the effects of SD diet through the second generation (F2) of *MsrA* −/− relative to WT mice with respect to their oxidized protein levels; activities and expression of seleno and antioxidant enzymes (cellulargluthathione peroxidase (GPx), Trr and selenoprotein P (SelP)) and the activities of the Msr-system related enzymes (MsrB, MsrA (in WT mice only), Trx, Trr and glucose-6-phosphate dehydrogenase (G6PD); that is involved in NADPH production). Following F1 generation of the SD diet, the mice are limited in their Se consumption only when they are weaned, as before then they acquire Se through their mother's milk. However, continuation of the SD diet through F2 generation will cause the Se deficiency to start at birth, as the mother's milk will be already deficient in Se. The current study will show in more detail the effects of Se deficiency, via the F2 generation of the SD diet, on the mouse antioxidant defense and how it is exacerbated in tissues of the *MsrA* −/− mice. Furthermore, it will demonstrate the importance of MsrA in regulating antioxidants and protein oxidation under prolonged Se deficiency. Thereby, supporting the importance of the Msr system in maintaining efficient antioxidant defense under conditions that cause lower activity of selenoproteins involved with scavenging of ROS.

Materials and methods

Materials

Trx antibodies were purchased from Abcam Inc, UK. Anti-G_6PD antibodies were purchased from Bethyl, Inc, US. Anti-SelP antibodies were generously provided by Dr U. Schweizer (Charite University Medical School, Berlin, Germany). Dimethylaminoazobenzene-4-sulfonyl (Dabsyl)-*R* or *S*-MetO was prepared by first making the *R* and *S* isoforms of MetO [1], followed by making the Dabsyl adduct, as described [4].

Animals and diets

The animals used in this study were the *MsrA* −/− mice and their parent strain (C57BL/129SvJ) [5], which served as the control. Weanling mice were fed a SD torula yeast-based diet or a SA diet containing 0.015 or 0.25 ppm Se as Na_2SeO_4, respectively. Diets were prepared by Zeigler based on their SD torula yeast diet (Zeigler Brothers, Gardner, PA). All nutrients, except Se, were provided in the basal diet at levels equal to those recommended by the American Institute of Nutrition *Ad Hoc* Committee on Standards for Nutritional Studies, 1997, 1980. The basal diet included the following components: amino acid composition (g/kg diet): L-arginine, 7.5; L-histidine·HCl·H_2O, 6.0; L-lysine·HCl, 16.5; L-tyrosine, 7.5; L-tryptophan, 2.5; L-phenylalanine, 7.5; L-threonine, 7.5; L-leucine, 11.0; L-isoleucine, 9.0; L-valine, 10.5; glycine, 6.0; L-proline, 4.5; L-glutamic acid, 40.0; L-alanine, 4.0; L-serine, 4.0; L-aspartic acid, 7.0; L-asparagine, 6.0; L-methionine, 8.2; and L-cystine, 3.5. Mineral composition (g/kg diet): K_2SO_4, 1.632; $CaCl_2 \cdot 2H_2O$, 1.05; Na_2CO_3, 1.5; MgO, 0.85; $Na_2SiO_3 \cdot 9H2O$, 0.254; ferric citrate (16% Fe), 0.22; manganese carbonate (43% Mn), 0.024; cupric carbonate (55% Cu), 0.011; sucrose, 14.44; in mg/kg diet: KIO_3, 0.35; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.371; $CrK(SO_4)_2 \cdot 12H_2O$, 9.625; H_3BO_3, 2.853; NaF, 2.223; nickel carbonate, 2.223; SnO, 0.25; NH_4VO_3, 0.462; $NaAsO_2$, 0.4; and zinc carbonate, 10.

Vitamin composition (mg/kg diet): nicotinic acid, 30; pantothenic acid, Ca salt, 16; pyridoxine·HCl, 7; thiamine·HCl, 6; riboflavin, 6; folic acid, 2; D-biotin, 0.2; vitamin B12 in 0.1% mannitol, 25; DL-α-tocopherol acetate (300 IU/g), 250; retinyl palmitate (250,000 IU/g), 16; vitamin D3 (400,000 IU/g), 2.5; vitamin K1, 1; and sucrose, 4638. Following 6 months on these diets, mice from each strain were inbred to produce F2 generation that were kept on the same diet for an additional 6 months. Groups of five mice of each strain at F1 or F2 generation were killed at 40 days immediately after weaning (21 days) and various tissues were removed and assays were performed. Extracts from all tissues were made in PBS, which contained protease inhibitor mixture (Roche, Gipf-Oberfrick, Switzerland). Parallel groups of mice were grown on the diets through 120 days and their weights were measured. All mouse experiments were conducted according to guidelines of the University of Kansas Animal Care and Use Committee.

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Determination of protein–MetO and carbonyl groups

Proteins from each extract were subjected to CNBr cleavage to quantify the proteinbound MetO [10]. CNBr cleaves peptide bonds on the carboxyl side of Met to yield homoserine but does not attack such bonds involving MetO [11]. Peptide hydrolysis with HCl and analysis of the resulting amino acids were carried out on samples before and after treatment with CNBr [12] and the amount of MetO was measured by integration of the corresponding peak. Protein carbonyl levels were determined by the reaction of the proteins in each extract with dinitrophenol followed by spectrometric measurements, as previously described [13,14].

Determination of MsrA *and MsrB activities*

Msr activity was measured by using 20 mM DTT, 200 μM Dabsyl-*R*-MetO and tissue protein extract. Following 30 min of incubation at 37°C, the separation and integration of the product peak were performed by using an HPLC method, as previously described [1,4,7].

The thioredoxin system, SelP, GPx, and G6PD activities and expression

Trx expression was monitored by a western blot procedure, using specific anti-Trx antibodies. Total Trr (Trr1 and Trr2) activity in cell extracts was measured using a colorimetric assay provided by Sigma. It is based on the reduction of 5, 5′-dithiobis (2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB) that produces a strong yellow color that is measured at 412 nm. SelP expression levels were detected by Western blot analysis using specific anti-SelP antibodies (limit of detection was about 1–2 ng of SelP). Similarly, the expression of G6PD was monitored using specific anti-G6PD antibodies. The specific activity of G6PD was measured using a commercial G6PD assay kit (Perkin Elmer Life and Analytical Sciences, Inc., USA). G6PD activity in each tissue extract was measured by monitoring the increase in the absorbance of NADPH produced (at 340 nm) to calculate the enzyme activity. Analysis for glutathione peroxidase (GPx) activity was performed according to the assay kit provided by ZeptoMetrix Corporation (Buffalo, New York). In this assay, cumene hydroperoxide is used as the peroxide substrate (ROOH) and glutathione reductase and NADPH are included in the reaction mixture. The formation of GSSG (glutathione) catalyzed by GPx is coupled to the recycling of GSSG back to GSH using glutathione reductase. NADPH is oxidized to $NADP^+$. The change in A_{340} due to NADPH oxidation is monitored and is indicative of GPx activity. Since all other reagents are provided in excess, the amount of GPx in the test sample is the rate-limiting factor. The oxidation of NADPH to $NADP^+$ is monitored spectrophotometrically by a decrease in absorbance at 340 nm (A_{340}). Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

Statistical analysis

Data presented are the mean ± standard deviation of the mean. Statistical analysis was carried out using paired or unpaired student's *t*-test and by one and two-way ANOVA to determine if the diets-dependent changes between the two mouse strains had a significant effect. A *p*-value of less than 0.05 was considered significant.

Results

Selenium deficient diet has a negative effect on body weight gain in MsrA − /− *mice*

MsrA − /− mice fed through their second generation (F2) with SD diet showed a slower growth rate in comparison to WT mice, as it is demonstrated by their weight gain through 120 days (Table I). Following 40 days of SD diet, the weight of the *MsrA* − /− mice was about 63% of their WT cohorts and reached compatible WT-weight by 120 days (Table I). In comparison, following SD through F1 generation or SA diet at F1 and F2 generations—no significant body weight changes between the WT and the *MsrA* − /− mice were observed (Table I). The lower body weight of the *MsrA* − /− mice in early life stages may indicate physiological alteration in regulation processes related to development of body growth, which are affected both by Se depletion and methionine oxidation.

Prolonged selenium deficient diet reduces the activity of MsrB in cerebellum of MsrA − /− *mice*

Several analyses were performed to assess the contribution of the MsrA to the antioxidants and Se functions in various tissues under SA or SD diet. Previously, we have shown that MsrA positively regulates the expression of the Se form of MsrB (MsrB1) [9]. Following the SD diet through F2 generation, mouse tissues from both strains were harvested after 40 days of the diet and the following observations were made.

The MsrB activity was unaffected by the SD diet except in kidneys and liver (Table II). The *MsrA* − /− did not affect the MsrB activity in most organs, except in cerebellum when SD diet had also continued through F2 generation and in kidneys and liver following F1 and F2 generations of the SD diet. The biggest effect on MsrB activity was shown to be in liver

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Table I. Body weight changes of *MsrA* − /− and WT control mice subjected to SA or SD diet.

	Total mouse body weight (g) following 40–120 days											
	SA diet (F2)				SD diet (F1)				SD diet (F2)			
	WT		*MsrA* − /−		WT		*MsrA* − /−		WT		*MsrA* − /−	
Days	F	M	F	M	F	M	F	M	F	M	F	M
40	17 ± 3	21 ± 3	16 ± 3	21 ± 3	17 ± 3	20 ± 3	16 ± 3	19 ± 3	16 ± 3	19 ± 3*	10 ± 3	12 ± 3*
80	21 ± 3	23 ± 3	21 ± 3	24 ± 3	20 ± 3	24 ± 3	20 ± 3	23 ± 3	21 ± 3	24 ± 2**	16 ± 3	20 ± 1**
120	26 ± 3	29 ± 3	27 ± 3	29 ± 3	28 ± 3	29 ± 3	28 ± 3	30 ± 3	27 ± 3	30 ± 3	27 ± 3	30 ± 3

Both mouse strains were fed through F1 and F2 generation with SA and SD diet (torula yeast-based diet) containing 0.25 or 0.015 ppm Se as Na_2SeO_4, respectively. Each value represents an averaged weight ($n = 5$). F1: F1 generation; F2: F2 generation. Following SA diet, the values obtained at F1 generation were similar to the values obtained at F2 generation for either mouse strain, respectively (data not shown). The ± numbers represent standard deviations. The significance of the lower MsrA − /− mouse weight relative to WT mouse weight is expressed by *p*-value. F, females; M, males. The symbol * indicates statistical significance of $p < 0.001$ and the symbol ** indicates statistical significance of $p < 0.05$ between the WT and the MsrA − /− mouse groups following SD diet at F2 generation.

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of the *MsrA* − /− mice fed with SA diet (MsrB residual activity was 32% of WT, respectively) (Table II). Additionally, the *MsrA* − /− mice exhibited ~50% reduction in their cerebellum MsrB activity relative to WT at the F2 generation of the SD diet (Table II). Throughout all the diet experiments no down regulation of the MsrB activity was observed in heart of both mouse strains, as the major source of MsrB activity in this tissue comes from the non-Se MsrB2 and MsrB3 [15,16].

Selenium deficient diet through F2 generation enhances protein oxidation especially in MsrA − /− *mice*

The biggest effect on protein–MetO accumulation was observed when shifting from SA diet to SD diet (Table III). Nevertheless, enhanced protein–MetO accumulations in the *MsrA* − /− mice relative to WT were shown in all tissues (~ × 1.5) and especially in lungs (× 2.0), following SD diet through F2 generation (Table III). In contrast, no significant differences in the levels of protein–MetO between the two mouse strains were detected at SD diet through F1 generation (except in cerebellum), while SA diet through F1 and F2 generations showed no detectable protein–MetO accumulations in both strains (Table III). Similarly, elevated levels of protein–carbonyl were detected in all *MsrA* − /− relative to WT mouse tissues at F2 generation of the SD diet and were mostly pronounced in brain-cerebellum (× 2.0), Lungs (× 2.0) and brain-cerebrum (× 1.5) (Table IV). No significant differences in the levels of protein–carbonyl between the two mouse strains were detected at SD diet through F1 generation, while following SA diet through F1 and F2 generations no protein–carbonyl accumulations were detected in both mouse strains (Table IV). The lack of detectable levels of protein–carbonyl following SA diet is mainly due to the fact that the mice tested were too young to accumulate significant amounts of protein–carbonyl

Table II. MsrB activitiy in various tissues of *MsrA* − /− and WT mice following SA or SD diet.

	MsrB specific activity (nmol Dabsyl–methionine/min/mg protein)					
	SA diet (F2)		SD diet (F1)		SD diet (F2)	
Tissue	WT	*MsrA* − /−	WT	*MsrA* − /−	WT	*MsrA* − /−
Cerebrum	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Cerebellum	1.6 ± 0.1	1.4 ± 0.2	1.5 ± 0.2	1.3 ± 0.2	1.4 ± 0.2¶	0.7 ± 0.1¶
Lungs	1.2 ± 0.2	1.1 ± 0.5	1.1 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.2
Heart	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Kidneys	7.0 ± 0.9**	4.0 ± 0.7**	3.0 ± 0.3	2.0 ± 0.2	3.0 ± 0.3	2.0 ± 0.2
Liver	19.0 ± 2.0*	6.0 ± 0.7*	3.0 ± 0.4	2.0 ± 0.3	3.0 ± 0.4	2.0 ± 0.3

Following 40 days of SA or SD diet through F2 generation MsrB activity was measured in various tissues of both mouse strains. The MsrB activity was measured by using 20 mM DTT, 200 μM Dabsyl-*R*-MetO and tissue protein extract. After 30 min of incubation at 37°C, the separation and quantitation of the product were performed by using an HPLC method, as previously described [1,4,7]. F1: F1 generation; F2: F2 generation. Following SA diet, the values obtained at F1 generation were similar to the values obtained at F2 generation for either mouse strain, respectively (data not shown). Each value represents an averaged activity ($n = 5$). The ± numbers represent standard deviations. The significance of the lower MsrB activity in the *MsrA* − /− mouse relative to WT mouse is expressed in *p*-value. The symbols *, ¶ and ** indicate statistical significance of $p < 0.001$ between the various pairs of WT and the *MsrA* − /− mouse groups following SA or SD diet at F2 generation, respectively.

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Table III. MetO accumulation in various tissues of WT and *MsrA* − /− mice following SA or SD diet.

	Percent protein–methionine as MetO					
	SA diet (F2)		SD diet (F1)		SD diet (F2)	
Tissue	WT	*MsrA* − /−	WT	*MsrA* − /−	WT	*MsrA* − /−
Cerebrum	N.D.	N.D.	9.0 ± 1.3	9.0 ± 1.2	9.0 ± 1.0*	14.1 ± 2.0*
Cerebellum	N.D.	N.D.	14.0 ± 2.4**	18.0 ± 2.2**	15.3 ± 1.6*	23.5 ± 1.5*
Lungs	N.D.	N.D.	15.1 ± 1.7	16.3 ± 2.1	15.1 ± 2.1*	28.0 ± 3.2*
Heart	N.D.	N.D.	16.0 ± 2.3	16.5 ± 1.5	16.2 ± 1.1*	25.3 ± 1.2*
Kidneys	N.D.	N.D.	8.0 ± 1.2	8.5 ± 1.6	15.1 ± 2.3*	23.0 ± 2.4*
Liver	N.D.	N.D.	6.0 ± 1.0	6.0 ± 1.8	13.3 ± 1.4*	20.2 ± 2.1*

Following 40 days of SD or SA diet, the protein–MetO content was measured in various tissues of the respected mice. Cellular protein extracts were treated with/out CNBr and their relative MetO levels were assayed using amino-acid analysis technique, as previously described in Ref. [4]. Each value represents an averaged percent MetO accumulation ($n = 5$). F1: F1 generation; F2: F2 generation. Following SA diet, the values obtained at F1 generation were similar to the values obtained at F2 generation for either mouse strain, respectively (data not shown). The ± numbers represent standard deviations. The significance of the higher protein–MetO levels in the *MsrA* − /− mouse relative to WT mouse is expressed in *p*-value. The symbol * indicates statistical significance of $p < 0.001$ and the symbol ** indicates statistical significance of $p < 0.05$ between the WT and the *MsrA* − /− mouse groups following SD diet at F2 or F1 generation, respectively. N.D., not detected.

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(40 days post-weanling). In summary, the biggest effect on protein carbonylation occurred when shifting from SA diet to SD diet with no additional effect in the second generation (including in liver and kidneys in this case). However, the *MsrA* knockout effect was shown for two tissues (brain and lungs) and only through the second generation of the SD diet.

These results clearly demonstrate that enhanced post-translational modification to proteins occurring following prolonged SD diet is predominantly expressed in brain and lungs of the *MsrA* − /− mouse. Correlative analysis between the MsrB activities and the tissue specific-protein–MetO and carbonyl accumulations in the *MsrA* − /− and WT mice imply a differential protection by MsrB and MsrA from oxidative damage. MsrB1 had a higher contribution in protecting the brain-cerebellum from protein–carbonyl accumulation through F2 generation of the SD diet (50% decrease of MsrB activity compared to × 2.0 of protein–carbonyl level in *MsrA* − /− relative to WT mice (Tables II and IV, respectively). Apparently, MsrA has a special protective role in preventing protein–MetO and carbonyl accumulations in lungs and brain of *MsrA* − /− mice through F2 generation of the SD diet (*MsrA* − /− mice exhibited only 10% decrease in MsrB activity and no MsrA activity) in comparison to × 1.5–2.0 of protein–MetO and carbonyl levels (Tables II–IV).

Selenium deficient diet through F2 generation reduces thioredoxin reductase activity and induces glucose 6-phosphate dehydrogenase levels especially in MsrA − /− *mice*

The Msr activity is dependent on reduced Trx that participates in the conversion MetO to methionine. By its action, Trx is oxidized and then recycled into its reduced form by the Trr. The mammalian Trr is

Table IV. Protein–carbonyl accumulation in various tissues of WT and *MsrA* − /− mice following SA or SD diet.

	Protein–carbonyl (nmol carbonyl/mg protein)					
	SA diet (F2)		SD diet (F1)		SD diet (F2)	
Tissue	WT	*MsrA* − /−	WT	*MsrA* − /−	WT	*MsrA* − /−
Cerebrum	N.D.	N.D.	4.0 ± 1.0	4.0 ± 1.0	4.0 ± 1.0*	6.0 ± 1.0*
Cerebellum	N.D.	N.D.	4.0 ± 0.8	4.0 ± 0.9	4.0 ± 1.0**	8.0 ± 1.0**
Lungs	N.D.	N.D.	2.0 ± 0.3	2.0 ± 0.2	2.0 ± 0.3**	4.0 ± 1.0**
Heart	N.D.	N.D.	6.0 ± 2.3	16.5 ± 1.5	16.2 ± 1.1*	25.3 ± 1.2*
Kidneys	N.D.	N.D.	3.0 ± 0.4	3.0 ± 0.3	3.0 ± 0.2*	4.0 ± 0.7*
Liver	N.D.	N.D.	3.0 ± 0.4	3.0 ± 0.3	3.0 ± 0.4*	4.0 ± 0.9*

Following 40 days of SD or SA diet, the protein–carbonyl content was measured in various tissues of the respected mice. Protein–carbonyl levels were determined by reacting the proteins from each extract with dinitrophenol followed by spectrometric measurements, as previously described in Refs. [15,16]. Each value represents an averaged protein–carbonyl accumulation ($n = 5$). F1: F1 generation; F2: F2 generation. Following SA diet, the values obtained at F1 generation were similar to the values obtained at F2 generation for either mouse strain, respectively (data not shown). The ± numbers represent standard deviations. The significance of the higher protein–carbonyl levels in the *MsrA* − /− mouse relative to WT mouse is expressed in *p*-value. The symbol * indicates statistical significance of $p < 0.08$ and the symbol ** indicates statistical significance of $p < 0.001$ between the WT and the *MsrA* − /− mouse groups following SD diet at F2 generation. N.D., not detected.

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Table V. Trr activity in various tissues of WT and *MsrA* − /− mice following SA or SD diet.

Trr specific activity (mU/min/mg protein)						
	SA diet (F2)		SD diet (F1)		SD diet (F2)	
Tissue	WT	*MsrA* − /−	WT	*MsrA* − /−	WT	*MsrA* − /−
Cerebrum	17.0 ± 2.0	17.5 ± 3.0	14.0 ± 1.0	15.0 ± 1.0	12.0 ± 2.0*	5.0 ± 1.0*
Cerebellum	8.0 ± 1.0	8.0 ± 1.0	7.0 ± 2.0	7.0 ± 2.0	6.0 ± 2.0*	1.0 ± 0.1*
Lungs	2.0 ± 0.2	2.1 ± 0.5	1.5 ± 0.1	1.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
Heart	1.8 ± 0.4	1.9 ± 0.5	1.6 ± 0.3	1.5 ± 0.2	1.0 ± 0.1	0.9 ± 0.1
Kidneys¶	98.0 ± 3.0	95 ± 4.0	50 ± 4.0	47 ± 4.0	14.0 ± 2.0*	6.0 ± 0.1*
Liver¶	42.0 ± 3.0	41 ± 4.0	15.0 ± 2.0	14 ± 2.0	4.0 ± 0.2*	1.0 ± 0.1*

Trr specific activity was measured in tissues of mice following 40 days of SA or SD diet. Trr activity in cellular protein extracts was measured using a colorimetric assay provided by Sigma. 1 mU represents the $\Delta A_{412\,nm}$/minute/mg protein.). Each value represents an average activity in five animals. F1: F1 generation; F2: F2 generation. Following SA diet, the values obtained at F1 generation were similar to the values obtained at F2 generation for either mouse strain, respectively (data not shown). Each value represents an averaged activity ($n = 5$). The ± numbers represent standard deviations. The significance of the lower Trr activity levels in the *MsrA* − /− mouse relative to WT mouse is expressed in *p*-value. The symbol * indicates statistical significance of $p < 0.001$ between the WT and the *MsrA* − /− mouse groups following SD diet at F2 generation. ¶, Only in kidneys and liver there was a significant general reduction of Trr activity (regardless of the mouse strain) in SD diet relative to SA diet, ($p < 0.001$).

a selenoprotein that its expression and activity are Se-dependent. In addition, MsrA positively regulates the expression of Trr in yeast and mouse tissues following hyperoxic conditions [5]. Therefore, it was interesting to determine the tissue-distributed Trr activity in *MsrA* − /− relative to WT mice in response to the SD diet. SD diet strongly affected the Trr activity in kidneys and liver (with a decrease in Trr activity observed in the SD diet), but had only moderate effects on other tissues (Table V). MsrA knockout had no effect on Trr activity unless the mice were also fed SD diet through F2 generation and the effect (a decrease in Trr activity) was shown in brain, kidneys and liver, but not in lungs and heart (Table V). No significant differences in the levels of Trr activity between the two mouse strains were detected at SD diet through F1 generation and SA diet through F1 and F2 generations (Table V). Additionally, no significant difference was observed in Trx expression between WT and *MsrA* − /− among all tissues examined following SA or SD diets, as determined by western blot analysis using specific antibodies against mammalian Trx (data not shown).

Trr uses the reducing agent NADPH for the reduction of Trx. The pentose phosphate pathway generates the NADPH that acts as a substrate for Trr. The first step of the oxidative branch of the pentose phosphate shunt is catalyzed by G6PD; thereby, its activity and expression levels were monitored in various mouse tissues following the SD diet. Neither diet alteration nor abolishing MsrA had any effect alone on G6PD activity. However, following SD diet through F2 generation in the *MsrA* knockout mice, significant increases in G6PD activity were observed in several tissues (Table VI). The activities (Table VI) and expression levels (Figure 1) of G6PD were significantly elevated in *MsrA* − /− hearts (× 5 compare to WT) lungs (× 2 compare to WT) and cerebellum (× 1.26 compare to WT) while very little differences were observed in brain tissues (Table VI and Figure 1).

Table VI. G6PD activity in various tissues of WT and *MsrA* − /− mice following SA or SD diet.

G6PD activity (mU/min/mg protein)						
	SA diet (F2)		SD diet (F1)		SD diet (F2)	
Tissue	WT	*MsrA* − /−	WT	*MsrA* − /−	WT	*MsrA* − /−
Cerebrum	5.5 ± 0.2	5.0 ± 0.2	6.1 ± 0.2	5.5 ± 0.2	5.1 ± 0.2	6.0 ± 1.0
Cerebellum	33.0 ± 7.0	35.0 ± 6.0	35.3 ± 6.0	36.4 ± 8.0	38.0 ± 8.0*	48.1 ± 6.0*
Lungs	9.3 ± 0.3	10.0 ± 0.4	10.5 ± 0.5	11.2 ± 0.5	10.0 ± 0.5**	20.1 ± 2.0**
Heart	9.0 ± 0.4	7.7 ± 0.4	10.0 ± 0.5	9.2 ± 0.5	8.1 ± 0.5**	40.3 ± 7.0**

G6PD activity was measured in tissues of mice following 40 days of SA or SD diet. The increase in the absorbance of NADPH produced was measured at 340 nm to calculate the enzyme activity. 1 mU represents the $\Delta A_{340\,nm}$/minute/mg protein. Each value represents an averaged activity ($n = 5$). F1: F1 generation; F2: F2 generation. Following SA diet, the values obtained at F1 generation were similar to the values obtained at F2 generation for either mouse strain, respectively (data not shown). The ± numbers represent standard deviations. The significance of the higher G6PD activity levels in the *MsrA* − /− mouse relative to WT mouse is expressed in *p*-value. The symbol * indicates statistical significance of $p < 0.05$ and the symbol ** indicates statistical significance of $p < 0.001$ between the WT and the *MsrA* − /− mouse groups following SD diet at F2 generation.

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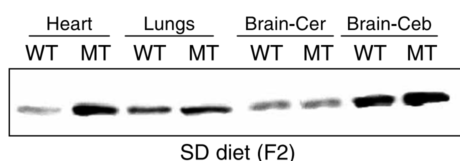

Figure 1. G6PD expression levels in various tissues of WT and *MsrA* − /− mice following SD diet. The G6PD level of expression was measured in tissues of mice following 40 days of SD diet through F2 generation. Expression of G6PD as it is detected by western blot analysis using specific antibodies against G6PD. About 20 μg per tissue extract was loaded in each corresponding lane. Brain-Cer, brain cerebrum; brain-Ceb, brain cerebellum; WT, wild-type mice; and MT, *MsrA* − /− mice. Each tissue contained pooled protein extracts from five animals.

Following SD diet through F1 generation and SA diet through F1 and F2 generations no significant differences were observed in the G6PD activity between the WT and *MsrA* − /− mouse strains (Table VI). In summary, the activities and expression levels of the Msr system components vary between the two mouse strains following SD diet through F2 generation and these changes are tissue specific.

Selenium deficient diet through F2 generation decreases GPx activity in brain and increases cellular SelP expression in tissues of the MsrA − /− *mice*

Among the known selenoproteins are the cellular GPx and the secreted SelP that are suggested to contribute to the cellular antioxidant defense mechanism. Consequently, it was interesting to monitor their expression/activity levels in response to the SD diet in the *MsrA* − /− mice. GPx is an enzyme found in cytoplasmic and mitochondrial fractions of cells. GPx catalyzes the reduction of hydrogen peroxide and hydroperoxides formed from fatty acids, thereby effectively removing toxic peroxides from living cells. It plays the important role of protecting cells from potential damage by free radicals, formed by peroxide decomposition. SD diet effects on GPx were extremely strong and progressive through F1 and F2 generations, with activity decreasing due to Se deficiency particularly for lungs, heart, kidneys and liver (Table VII). No additional decreases in GPx activity were shown in F2 generation with MsrA knockout mice for these tissues except for a small but significant activity decrease in liver (Table VII). On the other hand, brain was strikingly different than the other organs; SD diet had little or no effect on GPx activity alone, but in conjunction with *MsrA* knockout did have a significant effect in decreasing GPx activity. Except in brain, SD diet effects were much stronger than *MsrA* knockout effects (Table VII). It is suggests that MsrA is positively affecting the activities of both Trr and GPx while negatively affecting the activity and expression of G6PD at certain tissues, following prolonged SD diet (as it is demonstrated in Tables V–VII and Figure 1).

SelP is an abundant extracellular glycoprotein that is rich in selenocysteine. Evidence supports functions of the protein in Se homeostasis and oxidant defense [17]. *SelP* knockout mice have very low Se concentrations in the brain and testis, with severe pathophysiological consequences in each tissue [18–20]. Following SD diet through F2 generation, the plasma levels of SelP decreased significantly in both mouse strains to below the level of detection (Figure 2A). Similar results were obtained with mice fed SD diet through F1 generation (data not shown). No evidence has been presented yet that intracellular pools of it exist, aside from those associated with its synthesis and degradation. Indeed very low levels of SelP were

Table VII. GPx activity in various tissues of WT and *MsrA* − /− mice following SA or SD diet.

	GPx activity (nmol NADPH/min/mg protein)					
	SA diet (F2)		SD diet (F1)		SD diet (F2)	
Tissue	WT	*MsrA* − /−	WT	*MsrA* − /−	WT	*MsrA* − /−
Cerebrum	45 ± 6	47 ± 5	38 ± 5	36 ± 5	35 ± 4*	13 ± 1*
Cerebellum	25 ± 2	24 ± 3	20 ± 3	18 ± 2	22 ± 3*	9 ± 2*
Lungs¶	230 ± 25	220 ± 30	150 ± 17	140 ± 20	10 ± 1	7 ± 1
Heart¶	60 ± 7	57 ± 6	40 ± 5	38 ± 4	12 ± 1	10 ± 2
Kidneys¶	160 ± 25	170 ± 35	100 ± 15	105 ± 17	18 ± 1	20 ± 2
Liver¶	320 ± 40	315 ± 50	160 ± 20	150 ± 18	22 ± 2**	14 ± 1**

GPx activity was measured in tissues of both mouse strains following 40 days of DA or SD diet. GPx activity was performed according to the assay kit provided by ZeptoMetrix Corporation (Buffalo, New York). In this assay, cumene hydroperoxide is used as the peroxide substrate (ROOH) and glutathione reductase and NADPH are included in the reaction mixture. The change in A_{340} due to NADPH oxidation is monitored and is indicative of GPx activity. Each value represents an averaged activity ($n = 5$). F1: F1 generation; F2: F2 generation. Following SA diet, the values obtained at F1 generation were similar to the values obtained at F2 generation for either mouse strain, respectively (data not shown). The ± numbers represent standard deviations. The significance of the lower GPx activity levels in the *MsrA* − /− mouse relative to WT mouse is expressed in *p*-value. The symbol * indicates statistical significance of $p < 0.001$ and the symbol ** indicates statistical significance of $p < 0.05$ between the WT and the *MsrA* − /− mouse groups following SD diet at F2 generation. ¶, Only in kidneys, liver, heart and lungs there were a significant general reduction of Trr activity (regardless of the mouse strain) in SD diet relative to SA diet, ($p < 0.001$).

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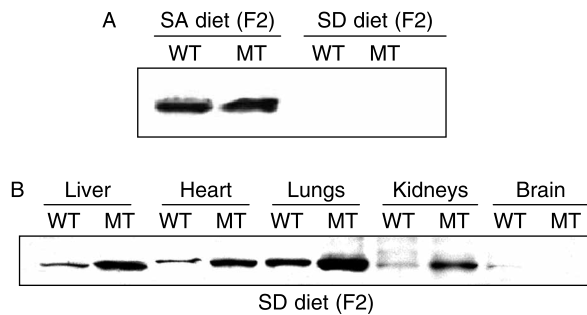

Figure 2. Expression of SelP in various tissues and plasma of WT and *MsrA* − /− mice following SD diet. The SelP expression was measured in tissues of mice following 40 days of SD diet through F2 generation. A, Western blot analysis of plasma proteins (pooled from five animals for statistical significance) using specific antibodies against mammalian SelP following SA or SD diet. The molecular weight of the protein band was ~55 kDa which corresponded to the major glycosylated form of SelP; and B, Western blot analysis of protein extracts taken from various tissues (pooled from five animals for statistical significance) using specific antibodies against mammalian SelP. The molecular weight of the protein band was ~45 kDa which correspond to the nonglycosylated form of SelP. In both A and B panels: WT, wild-type mice; MT, *MsrA* − /− mice. About 20 μg per tissue extract was loaded in each corresponding lane.

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detected in tissues of WT mice subjected to SD diet through F2 generation (Figure 2B). In addition, following SD or SA diet through F1 generation, no detectable intracellular levels of SelP in tissues of both mouse strains were observed (data not shown). However, unpredictably, the cellular levels of SelP in the *MsrA* − /− mouse tissues were elevated compare to the WT mouse tissues except for brain following SD diet through F2 generation, respectively (× 5 for liver and heart, × 4 for lungs and × 3 for kidneys with very low detection in brains of both mouse strains) (Figure 2B). Among the possible explanations for the latter increased cellular level of SelP in the *MsrA* − /− mouse tissues is either enhanced synthesis or lower degradation rate of the protein or both. Another possibility is a reduced level of SelP secretion rate in the *MsrA* − /− mouse tissues (Figure 2A). This data may imply a relevance of the SelP antioxidant properties in an MsrAcompromised system (See “Discussion”).

Discussion

Following SD diet at F1 generation, no significant observed phenotype has been noted in the *MsrA* − /− relative to WT mouse, except for enhancing its atypical walking pattern (“tip toe” walking) [9]. However, continuing the SD diet through F2 generation caused slower body weight gain in *MsrA* − /− mice till 120 days of the diet (that started immediately after weanling) (Table I). This phenotype may imply that a significant decrease in the levels of certain selenoproteins (including MsrB1 and Trr) in conjunction with the absence of the MsrA protein may interfere with normal body growth at early stages of development. The observed phenotype may be related to the insufficient reduction of MetO residue/s in key proteins participating in early stages of mouse growth. It will be of great interest to identify the specific proteins in which their methionine/s oxidation alter their ability to regulate directly or indirectly the function of growth related enzymes.

The MsrA protein positively regulates Trr expression (especially under oxidative stress conditions) [5] and MsrB1 (under non-stress conditions) [9]. Following SA diet, the most significant decrease in MsrB activity observed was mostly in liver and kidney tissues of *MsrA* − /− mice (Table II). However, only following SD diet through F2 generation, the *MsrA* − /− mouse cerebellum showed a dramatic decline in MsrB activity in comparison to WT brain-cerebellum (Table II). Accordingly, it is suggested that a long-term SD diet exacerbates the negative effect on MsrB expression in the absence of MsrA only in cerebellum. It is possible that in cerebellum, the expression levels of MsrA and Se tightly regulate the expression level of MsrB to avoid excess MsrB synthesis when the potential for full reduction of protein−*S*-MetO is limited. Accordingly, high level of *S*-MetO and low level of Se may be the secondary signals for reducing the synthesis of MsrB.

It is apparent, that enhanced protein oxidation occurs in the *MsrA* − /− mouse tissues following SD diet through F2 generation, in comparison to F1 generation and more so relative to WT mouse tissues, respectively (Tables III and IV). The MsrA protein is highly abundant in brain cerebellum and alveolar macrophages in lungs [21]. Both cerebellum and alveolar macrophages require a high level of antioxidant defense to maintain their proper function. It is hypothesized that lower levels of Se reduce the basal transcriptional level of selenoproteins with antioxidant properties (including MsrB) and consequently lead to a compromised antioxidant defense resulting in enhanced protein oxidation. Based on the current observations, it is concluded that lack of MsrA exacerbates the effects caused by prolonged Se deficiency.

The mammalian Trr enzyme is a selenoprotein that plays an important role in antioxidant defense and also a major component of the Msr system. Moreover, MsrA is positively up regulating Trr expression under oxidative stress conditions [5]. As a result, both mouse strains that were subjected to prolonged SD diet (through F2 generation) demonstrated a dramatic decrease in their Trr activity mainly in liver and kidneys (Table V). The observed effects of the SD diet are again much stronger than the *MsrA* knockout effects, except in brain where the opposite is true (when both *MsrA* − /− mice and SD diet are applied through F2 generation—the effect of MsrA absence

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is stronger) (Table V). One possible explanation is that very low Se levels in brain contribute to the enhancement of oxidative stress that in turn is negatively affecting Trr expression especially in the absence of MsrA; similarly to the effect of hyperoxia on Trr levels in *MsrA* − /− mice (Moskovitz et al. 2001). Consequently, lowering Trr activity by both dietary selenium and lack of MsrA may play a significant role in fostering oxidative damage to proteins (as it is shown in Tables III and IV).

The reduction of Trx by Trr requires NADPH. The major supplier of NADPH is the pentose phosphate pathway initiated by G6PD. Cells lacking G6PD are more sensitive to cell death that is mediated by oxidative stress [22,23]. As shown in Table VI, hearts and lungs of *MsrA* − /− mice that were subjected to the SD diet through F2 generation have significantly elevated expression and activity of their G6PD, relative to control WT mice. Both lungs and heart are the first organs that are exposed to high level of oxygen. Thus, it is essential to maintain sufficient reduction power (like NADPH) to prevent premature cell-death in response to extensive oxidative-stress conditions. It is possible that the elevation of G6PD in these tissues (subjected to Se deficiency) serves as a compensation mechanism for the lower antioxidant defense system in the *MsrA* − /− mice, under conditions of prolonged SD diet. It is important to note that neither SD diet nor lacking of MsrA alone showed the effect. The mechanism in which the up-regulation of G6PD occurs in the *MsrA* − /− mice (Table VI) is yet to be discovered. It may be that the signal-mediators for this phenomenon are a combination of certain levels of cellular Se and MetO (as free or protein-bound) that initiate signal transduction cascade leading to higher transcription level of *G6PD.*

One selenoprotein that is considered to be a good marker for Se deficiency is the secreted protein SelP [17]. Indeed, following SD diet through F2 generation, both mouse strains exhibited very low levels of plasma SelP that was below the assay's limits of detection (Figure 2A). However, contrary to expectations, the cellular levels of SelP were significantly higher in the *MsrA* − /− mice compared to control (Figure 2B). Usually, the SelP protein is secreted from cells to the plasma and one of its possible roles is to deliver Se to tissues via its rich selenocysteine residues content [17,24]. Another possible function of SelP is to act as an antioxidant by its potential reducing activity. SelP contains several redox centers in the form of cysteine and selenocysteine residues. Saito et al. [25] have shown that the human SelP can catalyze the oxidation of reduced gluthathione (GSH) by a phosphatidylcholine hydroperoxide. Moreover, Trx was found to be a very good substitute for GSH as a reducing agent [26]. If indeed the elevation of SelP level in the *MsrA* − /− mice reflects a compensatory mechanism to accommodate higher cellular oxidative toxicity, it will suggest that SelP may have an important role as an antioxidant. Finally, support for this study's observations regarding the level of expression of the glycosylated form of the cellular SelP and nonglycosylated form of SelP in plasma and its possible role in protecting against oxidative-stress comes from the recent finding demonstrating SelP function as an antioxidant [27]. Therefore, it will be interesting to crossbreed the *MsrA* − /− mouse with *SelP* − /− mouse to create a double *MsrA* − /−/-*SelP* − /− mouse and monitor for abrogated phenotypic characterization of either original source parent mouse (*MsrA* − /− or *SelP* − /− mice). It is expected that oxidative-stress related phenotypes will be enhanced in the *MsrA* − /−/*SelP* − /− mice relative to each of the parent strain.

Taken all together, the current study shows that the *MsrA* − /− mice shows significant oxidative damage to proteins as a consequence of long term SD diet through F2 generation. Additionally, the enhanced accumulation of post-translational modifications in the *MsrA* − /− mice may be due to a compromised antioxidant defense. Not all tissues are equally affected by the Se restriction. However, to compensate for the partial loss of selenoproteins that function also as antioxidants, certain tissues may up-regulate the activity/expression of specific proteins that are involved in reduction or peroxidation processes (like G6PD and SelP, respectively).

Unlike SelP and more like Trr, the activity of the selenoprotein and antioxidant enzyme GPx sharply declined in brains and livers of *MsrA* − /− mice subjected to the SD diet Through F2 generation (Table VII). One possible explanation is that similarly to Trr, GPx expression is also under the control of MsrA especially under oxidative stress conditions mediated by prolonged Se deficiency. The levels of GPx were not dramatically altered following F1 generation of the SD diet compare to the F2 generation (up to ~50%, depending on the tissue) probably due to the relatively short time of the diet administration right after weaning. The F2 generation of the SD diet enabled a dramatic effect of the SD diet and it is likely due to deficiency of Se during the weaning period.

Among all tested tissues only brain-cerebellum showed a major combined decrease in specific activities of MsrB, Trr and GPx. This observation may reflect a possible cerebellum mal-function associated with enhanced oxidative stress. It has been already noted that *MsrA* − /− mice exhibited an "a-typical tip-toe" walking pattern that is exacerbated as a consequence of SD diet [5,9]. Since the cerebellum is also responsible for certain motor functions, it is possible that this form of ataxia is at least partially due to the significant loss of the above antioxidant activities. Further investigations are underway to determine the identity of the oxidized

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proteins resulting from the SD diet. The gathered data will enable us to follow signal transduction pathways and/or key proteins that are involved in the cellular regulation of oxidative-stress that is associated with Se metabolism.

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