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Methionine Sulfoxide Reductase A Mediates Dietary Restriction-Induced Lifespan Extension in *Caenorhabditis elegans*

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Abstract

Background: Methionine sulfoxide reductase A (MsrA) is a well-studied antioxidant enzyme that has been found to be important for protecting cells against oxidative damage and regulating lifespan in several species. However, the role of MsrA in dietary restriction has not been examined. The authors evaluated the function of MsrA in dietary restriction-induced lifespan extension in *Caenorhabditis elegans*.

Methods: *C. elegans* loss-of-function *msra* mutant animals and wild type control animals were subjected to two widely used dietary restriction treatments, solid dietary restriction (sDR) and dietary restriction by liquid bacteria (BDR). The survival of the animals was evaluated and the data was statistically analyzed.

Results: The loss-of-function mutation of *msra* significantly suppressed the lifespan extension conferred by solid dietary restriction. By contrast, *msra* was dispensable for lifespan extension resulting from dietary restriction by diluted bacteria in liquid.

Conclusion: *msra-1* is a major factor in the sDR-induced lifespan extension. This result, coupled with the previous finding that MsrA mediates the effect of insulin-like signaling on lifespan extension, indicates an essential role of MsrA in the aging process in *C. elegans*.

Keywords: MsrA; Dietary restriction; Aging; Lifespan; *C. elegans*

Introduction

Dietary restriction (DR) is defined as a decrease in the nutrient uptake of an organism without causing malnutrition. DR is the most consistent and reproducible method used to increase lifespan and has been shown to do so in over twenty different species [1-3]. Although the effect of DR on life span has been extensively studied the exact mechanism by which DR acts is still not fully understood. However, there is considerable evidence that the lifespan extension seen in DR is due to a reduction in oxidative damage [4]. The modified free radical theory of aging proposed that reactive oxygen species (ROS) derived from oxygen are responsible for cellular damage associated with ageing. This could be due to a decrease in the generation of reactive oxygen species (ROS) and/or an increase in the cellular protective mechanisms against oxidative damage under conditions of DR. As example, rodents subjected to DR showed a decrease in the age-associated production of mitochondrial ROS and slower accumulation of oxidative damage [4]. In addition, there are several reports of life span extension, independent of DR, that have resulted from over-expression of enzymes such as superoxide dismutase (SOD) [5], catalase [6] and MsrA [7], which are known to protect cells against oxidative damage either by destroying ROS or, as in the case of MsrA, repairing damage to proteins due to methionine oxidation [8]. The MSR system is unique in that it can also function as part of an ROS scavenger system in which methionine residues in proteins can function as catalytic antioxidants [9].

The methionine sulfoxide reductase system is a highly conserved system [10]. Methionine residues are very susceptible to oxidation by ROS and are converted to either the S epimer of methionine sulfoxide (Met-S(o)) or the R-epimer (Met-R(o)) when chemically oxidized by ROS. The methionine oxidation can be reversed by the methionine sulfoxide reductase system, MsrA and MsrB [8,10]. MsrA reduces the S epimer of Met(o) in proteins and MsrB is specific for the R epimer of Met(o) [11-15].

Several studies have provided evidence that MsrA is important in protecting cells against oxidative damage and in the aging process. Eschericia coli and yeast mutants lacking MsrA have an increased sensitivity to oxidative stress [16-18]. Compared to wild type mice, MsrA-/- mice also show decreased resistance to oxidative stress [19,20]. Moreover, expression of both MsrA and MsrB declines in senescent human fibroblasts cells compared to young cells, and this decline is associated with accumulation of oxidized proteins [21]. Interestingly, over-expression of MsrA lowers the levels of ROS in cells [22] and increases lifespan in fruit flies and yeast [7,23].

In *C. elegans*, axenic medium imposes dietary restriction (ADR) and extends *C. elegans* lifespan [24]. ADR was found to cause higher activities of the antioxidant enzymes SOD and catalase [24]. *C. elegans* fed with a dilution of bacteria in liquid medium (bacterial dietary restriction: BDR) showed an increased lifespan, which was dependent on PHA-4, a FOXA transcription factor [25]. Interestingly, PHA-4 was found to mediate BDR-induced longevity by upregulating SOD [25]. Recently, a *C. elegans* homolog of MsrA has been identified [26] and an *msra* deletion mutant was reported to be sensitive to oxidative stress [27]. Moreover, this deletion mutation of *msra* decreases the lifespan of the long-lived *daf-2* mutant worms [27]. Interestingly, the DAF-16/FOXO3 transcription factor, which is a major target of the *daf-2*

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insulin-like signaling pathway, positively regulates *msra* expression [27]. However, the role of MsrA in DR has not been examined. Here we show that MsrA mediates the effect of solid dietary restriction on lifespan extension in *C. elegans*.

Materials and Methods

C. elegans strains

The Bristol N2 strain is the wild type *C. elegans* strain used in all experiments. Strains were maintained at 20°C as described by Brenner [28]. To obtain the homozygous *msra-1(tm1421)* mutant, N2 males were crossed to the *msra-1(tm1421) II; lin-15B(n765ts)* mutants. The outcross progeny were selected and self-propagated to remove the *lin-15B(n765ts)* mutation. Fifty *msra-1* mutant worms were examined for the homozygous deletion mutation by following a standard single-worm PCR [29] and all tested worms showed the homozygous deletion. The sequences of the primers used were:

msra-1 F: 5' - CACATTTTGATTCCGCCCCGATT - 3';

msra-1 R: 5' - GACAACAACTCTTCAAATCCATT - 3'.

Solid dietary restriction (sDR) lifespan analysis

The sDR method was modified from that previously described [30]. Briefly, the OP50 overnight culture was washed and re-suspended in S Basal media without cholesterol. Serial dilutions were performed to achieve bacterial concentrations of 5.0×10^{11}, 5.0×10^{10}, 5.0×10^{9}, and 5.0×10^{8} bacteria/ml. 250 µl of these diluted bacterial cultures were spotted on each 60 mm plate on the day of transfer. 30 adult worms were placed on each plate containing various concentrations of bacteria starting at day 1 of adulthood. Three plates for each strain at each bacterial concentration were set up. In the first week of the lifespan experiments, DR plates that contain FUdR (20 µg/ml) were used to prevent progeny from hatching. Worms were transferred to fresh plates every other day. Dietary restriction (sDR) was considered 5×10^{8} bacteria/ml and *ad libitum* (AL) was 5×10^{11} bacteria/ml [30]. The nematode growth media (NGM) plates were modified by excluding peptone and increasing agar from 1.7% to 2.0%. Carbenicillin (50 µg/ml) was added to the plates to further prevent bacteria growth. The experiment was performed at 20°C.

Bacterial dietary restriction (BDR) lifespan analysis

BDR was performed as previously reported [25,31]. To prepare liquid cultures for dietary restriction, an OP50 overnight culture grown at 37°C was washed and resuspended to adjust the bacterial concentration to 1.5×10^{9} cells/ml in S Basal media containing cholesterol (5 mg/ml), carbenicillin (50 mg/ml), tetracycline (1 mg/ml) and kanamycin (10 mg/ml). Serial dilutions were performed to achieve bacterial concentrations of 7.5×10^{8}, 1.5×10^{8}, 7.5×10^{7}, 2.5×10^{7} and 5.0×10^{6} bacteria/ml. Dietary restriction (BDR) was considered 7.5×10^{7} bacteria/ml and *ad libitum* (AL) was 7.5×10^{8} bacteria/ml [25]. To prevent progeny reproduction, FUdR was added at a concentration of 20 µg/ml in cultures during the first twelve days of lifespan analysis.

To subject animals to dietary restriction, well-fed 1-day old adult worms were transferred into liquid cultures at different bacterial concentrations. Liquid cultures were done in 12-well cell culture plates containing 1 ml of culture per well. Each lifespan experiment consisted of 4 wells with 15 worms per well. The 12-well plates were placed on a gentle rocker at 20°C during the lifespan analysis. Survival of the animals was scored daily and worms were transferred to new cultures every 3-4 days. Animals were considered dead when they failed to respond to touch.

Statistical Analysis

GraphPad Prism 5 was used to generate survival curves and determine medians, means and percentiles. In all cases, P-values were calculated using the log-rank (Mantel– Cox) method.

Results and Discussion

Previous studies have shown that ADR and BDR extend *C. elegans* lifespan and up-regulate antioxidant enzymes [24]. PHA-4 (FOXA) was reported to mediate BDR- induced longevity by controlling SOD transcription [25]. Our goal was to determine whether MsrA may also be required for DR-mediated lifespan extension. A *C. elegans msra-1(tm1421)* deletion mutant has been isolated [27], and we initially confirmed the homozygous deletion in the *msra-1* mutant. Fifty *msra-1* mutant worms were analyzed by using single worm PCR with primers flanking the deletion site. As expected, a 171 bp deletion band was detected in all *msra-1(tm1421)* mutant worms, which confirmed the 908 bp deletion in the *msra-1(tm1421)* mutant (Figure S1).

Next, *the msra-1(tm1421)* mutant worms were subjected to dietary restriction to examine the possible role of *msra-1* in DR-mediated lifespan extension. Various dietary restriction protocols have been developed in *C. elegans* such as decreasing food availability by bacteria dilution in liquid culture (BDR) [31] and on solid medium (sDR) [30]. Since DAF-16 is required for sDR-mediated lifespan extension [30] and *msra-1* is a target gene of DAF-16 [27], we initially examined the role of *msra-1* in plate-based dietary restriction (sDR). Consistent with a previous report [30], the lifespan of N2 worms fed with 5×10^{8} bacteria/ml (DR) is significantly increased compared to that of animals fed with 5×10^{11} bacteria/ml (AL: *ad libitum*). The mean lifespan is increased by 38% (from 21.25 days to 28.88 days; P<0.0001, log-rank test) and the maximum lifespan is extended by 20 days (from 31 to 51 days) (Figure 1 and Table 1). The sDR- treated *msra-1* mutants have a slightly shorter (but significant) median life span than sDR-treated N2 animals (26.13 days v.s. 28.88 days, P=0.0002). However, the most striking effect seen with the msra-1 mutants was on the maximum lifespan. The maximum lifespan of sDR-treated msra-1 mutants is 15 days shorter than that of N2 worms (36 vs. 51 days) (Figure 1 and Table 1). It should be noted that the mean lifespan of sDR-treated msra-1 mutants is still increased

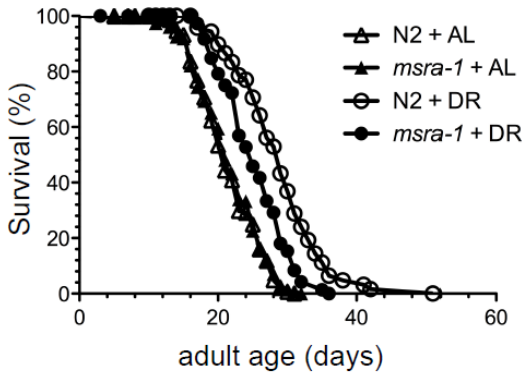

Figure 1: MSRA-1 mediates sDR-induced lifespan extension. Lifespan curves of wild- type N2 animals and *msra1* mutant worms treated by sDR (5×10^{8} bacteria/ml) and fed *ad libtum* (5×10^{11} bacteria/ml). N2 + sDR, mean lifespan 28.88 days and maximum 51 days; N2 + AL, mean 21.25 days and maximum 31 days; *msra-1* + sDR, mean 26.13 days and maximum 36 days; *msra-1* + AL, mean 21.38 days and maximum 32 days. This entire experiment was done twice and similar results were obtained. The pooled data from these two experiments are shown in this figure.

Table 1: Statistical analysis of lifespan data.

Strain and culture conditions	Mean lifespan (days)	Max.lifespan (days)	N[1]	P Value[2]
sDR				
N2 + 5×10^{11} cells/ml (AL)	21.25 ± 0.6291	31	145(31)	
N2 + 5×10^{8} cells/ml (DR)	28.88 ± 1.0078	51	63(44)	< 0.0001 (vs. N2 + AL)
msra-1 + 5×10^{11} cells/ml (AL)	21.38 ± 0.8985	32	142(29)	0.2083 (vs. N2 + AL)
msra-1 + 5×10^{8} cells/ml (DR)	26.13 ± 1.3901	36	72(24)	0.0002 (vs. N2 + DR)
BDR				
N2 + 7.5×10^{8} cells/ml(AL)	34.25 ± 3.7666	50	55(5)	
N2 + 7.5×10^{7} cells/ml (DR)	42.5 ± 1.8027	55	49(11)	0.0005 (vs. N2 + AL)
msra-1 + 7.5×10^{8} cells/ml(AL)	38.5 ± 1.6583	48	54(6)	
msra-1 + 1.5×10^{8} cells/ml(DR)	44.45 ± 1.5562	60	49(11)	< 0.0001 (vs. *msra-1* + AL)

[1]N = population size, numbers of censored animals are indicated in parenthesis;
[2]P values (log- rank test) for mean life span of each group compared to group indicated in parentheses.

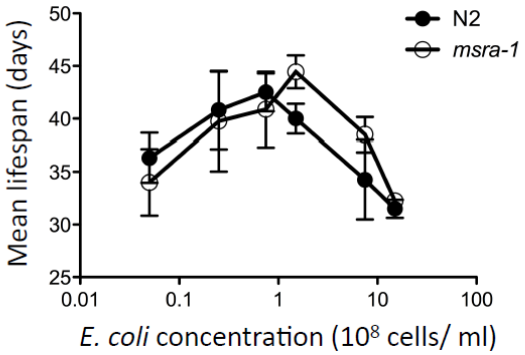

Figure 2: Dietary restriction using bacterial dilution (BDR) extends the lifespan of *msra-1(tm1421)* deletion mutants. BDR results in a parabolic curve for both wild-type worms (solid cycle) and *msra-1(tm1421)* mutant animals (open cycle). Error bars: s.e.m. The mean lifespan (44.45 days) of *msra-1* mutant at optimal DR (1.5×10^{8} bacteria/ml) is significantly longer than the mean lifespan (38.8 days) of worms fed *ad libitum* (7.5×10^{8} bacteria/ml) ($p < 0.0001$, log-rank test).

by 5 days compared to that of *msra-1* mutant worms fed ad libitum (from 21.38 days to 26.13 days, P<0.0001), suggesting that the *msra-1* mutation did not completely suppress the effect of sDR on the mean lifespan. As shown in Table 1 and Figure 1 the N2 and *msra-1* mutants have a similar lifespan when fed *ad libitum* (mean life spans are 21.25 and 21.38 days, respectively, and the maximum lifespans are 31 and 32 days, respectively). This is in contrast to a previous study showing that *msra-1* mutants lived 30% shorter than N2 [27] when fed *ad libitum*. We were concerned about the difference in the results so the lifespan of *msra-1* mutants fed ad libitum was examined in four independent trials and there was no obvious difference between N2 and *msra-1* mutant lifespans (Table S1). At present we cannot explain the different results obtained in the two studies. Regardless, the experiments demonstrate that the sDR-treated *msra-1* mutants have both a shorter median and maximum lifespan.

In contrast to sDR, BDR extends *C. elegans* lifespan independently of DAF-16. If *msra-1* mediates sDR-induced lifespan extension because it is a target gene of DAF-16, msra-1 might not be required for BDR to extend *C. elegans* lifespan. To test this, we subjected the *msra-1* mutant and control wild type N2 worms to BDR. In agreement with previous reports [25,32], wild-type N2 animals are short lived at both high and very low bacterial concentrations. However, N2 mean lifespan is significantly increased at the optimal bacterial concentration (Figure 2 and Table 1). Similar to N2 worms, the *msra-1* mutant worms also showed a parabolic curve with increased lifespan at the optimal bacterial concentration and short lifespan at both high and low bacterial concentrations (Figure 2 and Table 1). The results indicate that *msra-1* mutants respond to BDR similar to wild-type N2 worms, suggesting msra-1 is dispensable for lifespan extension induced by BDR. It should be noted that the optimal bacterial concentration to maximize lifespan extension for msra-1 mutants is 1.5×10^{8} cells/ml, but it is 7.5×10^{7} for N2 animals (Figure 2 and Table 1). Previous published studies have indicated that *C. elegans* with different genotypes can respond to DR differentially. Therefore, the concentration of bacteria in the food that maximizes lifespan for one genotype may be different from the one works for another genotype [32].

In *C. elegans*, dietary restriction has been mainly applied in four ways: dilution of bacteria in liquid medium (BDR) [31], *eat-2* mutants with pharyngeal defect and insufficient food intake [33], culture in axenic medium (ADR) [24] and dilution of bacteria on solid medium plate (sDR) [30]. All of these protocols extend *C. elegans* lifespan. It is of interest, and unexpected, that different forms of DR extend lifespan by different mechanisms [3,32,34]. For example, PHA-4, a FOXA transcription factor, is required for BDR-induced lifespan extension and for the longevity phenotype of *eat-2* mutants [25]. In contrast, although DAF-16, a different FOXO transcription factor, is dispensable for BDR-mediated lifespan extension, it is essential for sDR treatment to increase *C. elegans* lifespan [30]. It was suggested that different dietary restriction protocols may require different effectors for lifespan extension, possibly due to the fact that some nutrients may be more

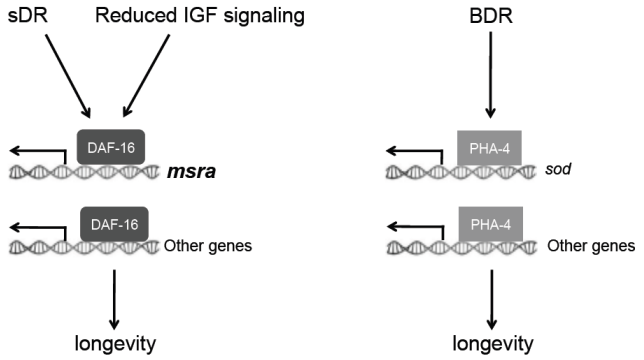

Figure 3: A model depicting a role of MSRA in dietary-restriction-induced longevity in *C. elegans*. As a target gene of DAF-16 FOXO transcription factor, MsrA is required for sDR-induced longevity, but is dispensable for BDR-mediated lifespan extension. In addition to MsrA, other target genes of DAF-16 are also required, especially for sDR- induced mean lifespan extension.

limiting than others depending on the DR method [34]. For example, sDR might reduce carbohydrates more severely than amino acids. Thus, sDR is dependent on FOXO that regulates carbohydrates metabolism. By contrast, another DR method BDR may mainly reduce amino acids. Therefore, BDR requires the FoxA/pha-4 transcription factor that has recently been found to be downstream of TOR (target-of-rapamycine) [35], the well-known amino-acid responsive pathway [36]. Interestingly, both PHA-4 and DAF-16 regulate transcription of the antioxidant enzyme superoxide dismutase [25]. BDR involves the PHA-4-dependent expression of *sod-1*, *sod-2* and *sod-5*. DAF-16 regulates the expression of *sod-1*, *sod-3* and *sod-5* in response to reduced *daf-2* insulin-like signaling [25].

Similar to SOD, expression of *msra-1* is also positively regulated by DAF-16 [27]. When the *daf-2* insulin-like signal is reduced or when *C. elegans* is under oxidative stress, the expression of *msra-1* is up-regulated dependent on DAF-16 [27]. The lifespan results presented here suggest that *msra-1* is a major factor in the sDR-induced lifespan extension because it is a target gene of DAF-16 (Figure 3), since both DAF-16 and MsrA are required for sDR, but not BDR, to extend *C. elegans* lifespan. Interestingly, dietary restriction was found to alleviate abnormal locomotor activity and dopamine levels of MsrA-/- mice, suggesting that DR can prevent some of the oxidative damage seen in MsrA-/- mice [37], although in another study using MsrA-/- mice there was no evidence of abnormal locomotor activity [20]. In addition, the influence of MsrA on yeast lifespan was independent of DR (growth in the presence of 0.5% glucose) [23]. Recently, it was reported that yeast forkhead box transcription factors FKH1 and FKH2, putative DAF-16 orthologs, are required for lifespan extension induced by severe caloric restriction (SCR) in which yeast cells are maintained in water [38]. However, the role of MsrA in these studies was not evaluated. The above results suggest different dietary restriction protocols may require different effectors for lifespan extension, as observed in *C. elegans*.

Since the mean lifespan of the sDR-treated *msra-1* mutant increased slightly, it appears that other *daf-16* target genes may also be required to achieve the full mean lifespan extension by sDR. It has been previously shown that *msra-1* accounts for most of the longevity of *daf-2* mutants, and *msra-1* is certainly the major factor involved in the maximum lifespan extension in the present studies using sDR. Thus, *msra-1*, as a target gene of the DAF-16 FOXO transcription factor, is essential for life span extension conferred by both dietary restriction and reduction of the *daf-2* insulin-like signaling pathway (Figure 3).

Conclusions

We provide genetic evidence that methionine sulfoxide reductase A (MsrA) plays an essential role in dietary restriction-mediated (sDR) lifespan extension in *C. elegans*. MsrA is an evolutionarily conserved antioxidant enzyme that has been found to be important for protecting cells against oxidative damage and regulating lifespan in several species. Interestingly, human Foxo3 positively regulates the expression of human MsrA and activates the *C. elegans msra-1* promoter in human HEK293 cells [27]. This suggests *msra-1* may be required for the health-beneficial effect of dietary restriction in other species including humans.

Summary

Dietary restriction has been shown to increase the lifespan of many species varying from yeast to rodents. Methionine sulfoxide reductase A (MsrA) is a well-studied antioxidant enzyme that has been found to be important for protecting cells against oxidative damage and regulating lifespan in several species. However, the role of MsrA in dietary restriction has not been examined. Recently, an ortholog of MsrA (Msra-1) has been identified in *Caenorhabditis elegans* and it is required for the longevity phenotype of a mutant with reduced insulin-like signaling. Here we show that a loss-of-function mutation of *msra-1* significantly suppresses the lifespan extension conferred by solid dietary restriction (sDR) in *C. elegans*. We also found that MsrA, like its positive regulator DAF-16/FOXO transcription factor, is dispensable for lifespan extension resulted from dietary restriction by diluted bacteria in liquid. These data suggest *msra-1* is a major factor in the sDR-induced lifespan extension because it is a target gene of DAF-16. This result, coupled with the previous finding that MsrA mediates the effect of insulin-like signaling on lifespan extension, indicates an essential role of MsrA in the aging process in *C. elegans*. Interestingly, human FOXO3a has been shown to regulate expression of *msra* and can bind to the *C. elegans msra-1* promoter, suggesting MsrA may be required for the health-beneficial effect of dietary restriction in other species including humans.

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