LIFE SPAN EXTENSIONS ASSOCIATED WITH UPREGULATION OF GENE EXPRESSION OF ANTIOXIDANT ENZYMES IN *CAENORHABDITIS ELEGANS*; STUDIES OF MUTATION IN THE AGE-1, PI3 KINASE HOMOLOGUE AND SHORT-TERM EXPOSURE TO HYPEROXIA.

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ABSTRACT

Life span could be modified by genetic or environmental perturbations in *Caenorhabditis elegans*. Here we show that two extensions of life span are associated with oxidative stress resistance and upregulation of the gene expression of antioxidant enzymes. First, mutations in *age-1* gene (PI3 kinase homologue) that confer life span extension, display oxidative stress resistance and increase in the gene expression of *sod-3*, one of two Mn-superoxide dismutases (SOD) and *ctl-1*, cytosolic catalase. In this study, these traits appear to be regulated by the following genetic pathway: *daf-2* (insulin receptor family) → *daf-18* (PTEN homologue) → *age-1* → *daf-16* (Fork head transcription factor family), similar to the genetic pathway for the life span extension. Second, we show that short-term exposure to hyperoxia extends life span slightly but significantly. This treatment increases oxidative stress resistance and the gene expression of three types of SOD isoforms. These results suggest that both of these two life span extensions are closely related with increase in the antioxidant defense function.

INTRODUCTION

Life span of metazoans can be modulated by environmental conditions: caloric restriction in various species of animals (1), low temperature in some poikilothermic animals (2) and low oxygen concentrations in the nematode (3). Life span could also be lengthened by genetic or environmental perturbations.

Life extension mutation

Single-gene mutations have been found to extend life span in *C. elegans*, *Drosophila* and mice (4). So far, the best-characterized system is an insulin-like signaling pathway that regulates life span of *C. elegans*. Mutations in *daf-2*, which encodes a homologue of the insulin receptor (5), and *age-1*, which encodes a homologue of the phosphatidylinositol-3-OH kinase (PI3 kinase) (6), and *pdk-1*, which encodes a PDK1, phosphoinositide-dependent kinase-1 homologue (7), extend life span (Age phenotype) (4). This phenotype is suppressed by mutations in *daf-16*, which encodes a Fork head transcription factor (8, 9, 10). Mutation in the *daf-18*, a homologue of PTEN phosphatase, suppressed Age

phenotype of *daf-2* (11, 12, 13, 14, 15). However, suppression of Age phenotypes of *age-1* by *daf-18* mutation was controversial; Dorman *et al.* reported complete suppression (16) but Larsen *et al.* reported partial suppression (11). Location of DAF-18 PTEN in the pathway for regulating life span is yet not clear.

It has been considered that activation of the DAF-2 insulin receptor by its ligand (17, 18, 19) leads to activation of AGE-1 PI3 kinase, which generates the phosphatidylinositol-3,4,5-triphosphate (PIP3), a second messenger (6). This second messenger activates the PDK-1 and AKT kinases (7, 20), which antagonize the activity of DAF-16 transcription factor. It seems likely that the insulin-like signaling pathway transcriptionally activates the expression of target genes that specify the efficient life-maintenance mechanism.

The aging theories which have been proposed thus far, can be investigated by examining the properties of these mutants, to determine whether the function of the gene network in *C. elegans* support or contradict them. The free radical theory of aging is attracting considerable attention (21, 22). Reactive oxygen species (ROS) such as $O_2^{\cdot-}$, H_2O_2 , and $OH^{\cdot}$, are generated during cellular metabolism, especially during mitochondrial energy production (23). These ROS, in turn, cause oxidative damage to DNA (24), and to proteins (25). This theory of aging proposes that, although the defense systems that enzymatically detoxify these ROS (23), as well as repair the oxidative damage (26), have evolved, not all ROS are detoxified; oxidative damage caused by the ROS not caught by the defense systems accumulates to cause deleterious consequences in senescence. The importance of oxidative stress in the aging processes is evidenced by the observation that overexpression of antioxidant defense enzymes in *Drosophila* extends life span (27, 28).

We have screened the Age mutants that are associated with the oxidative stress resistance phenotype, referred to as Oxr phenotype (29). The Oxr phenotype may be determined by pathways involved in the detoxification of ROS. These include superoxide dismutase (SOD), catalase, glutathione, and systems that repair oxidative damage. SOD is a major enzyme that protects against oxidative stress by catalyzing the removal of $O_2^{\cdot-}$ (23), a central ROS involved in the generation of various toxic ROS. In eukaryotes, there are three types of SODs: cytosolic CuZn-SOD; extracellular CuZn-SOD; and Mn-SOD, an enzyme located in the mitochondria, the major site of $O_2^{\cdot-}$ generation (23). There are several genes in *C. elegans* that encode SOD enzymes: *sod-1* encodes cytosolic CuZn-SOD (30), while *sod-2* and

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sod-3 each encodes Mn-SOD (31, 32, 33). We have shown that *daf-16* and *daf-18* act downstream of *daf-2* in the pathway for regulating oxidative stress resistance and *sod-3* expression similar to regulating life span (29). Here we demonstrate Oxr phenotype and increase in the expression of *sod-3* gene in the *age-1* mutants of *C. elegans* and also demonstrate the interactions between *age-1* and *daf-16* or *daf-18* in displaying these phenotypes.

Hormesis

Hormesis is a phenomenon which occurs when agents that are harmful in high doses or over long periods, actually produce beneficial effects such as life span extension when used in low doses or over short periods. *C. elegans* shows life-span-extension hormesis when exposed to low dose of radiation (34) or short-term heat exposure (35). We found that long-term hyperoxia shortened the life span of *C. elegans* (36). During the course of this investigation, we noticed slight extension of life span by normoxic incubation after hyperoxia (36). Darr and Fridovich, showed an adaptive induction of antioxidant defense in *C. elegans* (37). We explored the possibility that hyperoxia has hormetic effects in longevity. In the present study we found that short-term exposure of *C. elegans* to hyperoxia lengthen life span. There was also an adaptive response to hyperoxia that strengthened *C. elegans* against the lethal effect of paraquat-induced oxidative stress. We studied the gene expression of various antioxidant enzymes after hyperoxia.

RESULTS

Life span extension and oxidative stress resistance in *age-1* mutant

In order to elucidate the relationship between life span extension and ability of withstanding oxidative stress in *age-1* mutants, we compared life-span extension (Age) phenotype and oxidative stress resistance (Oxr) phenotype in *age-1* mutant and various double mutants of *age-1*.

Figure 1: Life span of *C. elegans* mutants. The number of animals: N2; 118, *age-1(m333)*; 87, *age-1(mg44)*; 76, *daf-16; age-1(m333)*; 78, *age-1(m333); daf-18*; 71, *daf-16*; 49, *daf-18*; 51.

We measured life span of *age-1* mutant and double mutants of *age-1*. As shown in Figure 1, two strains of *age-1*, *age-1(m333)* and *age-1(mg44)*, lived twice as long as wild type N2 strain. Life span of double mutant of *age-1* and *daf-16(m26)* was similar to the wild type indicating that a mutation in *daf-16* suppressed Age phenotype of the *age-1* mutant. Although the life span of double mutant of *age-1* and *daf-18(e1375)* was shorter than that of *age-1*, it was longer than that of the wild type indicating that *daf-18* did not fully suppress Age phenotype of the *age-1* mutant. These results confirmed the report of Larsen et al. (11).

Figure 2: Survival curves of *C. elegans* mutants under oxidative stress. The number of animals: N2; 80, *age-1(m333)*; 63, *age-1(mg44)*; 64, *daf-16; age-1(m333)*; 55, *age-1(m333); daf-18*; 60, *daf-16*; 90, *daf-18*; 93.

For Oxr assay, we examined the survival period of each mutant under experimentally-induced, acute oxidative stress. For the oxidative stress resistance assay, we used paraquat (PQ), an intracellular O_2^- generator (38), under 98% oxygen. Although 98% oxygen itself did not have a lethal effect on the wild type and the mutants in this study over 6 days (data not shown), it did stimulate PQ toxicity. Figure 2 showed that two *age-1* strains were more resistant to oxidative stress than the wild type (Oxr phenotype). The double mutant of *age-1* and *daf-16* was sensitive to oxidative stress similar to the wild type. Although the double mutant of *age-1* and *daf-18* was less resistant to oxidative stress than *age-1*, it was apparently more resistant to oxidative stress than the wild type. These results indicate that Oxr phenotype of *age-1(m333)* was suppressed by a mutation of *daf-16* but not completely suppressed by a mutation of *daf-18(e1375)*.

Level of antioxidant enzyme mRNA in the *age-1* mutant

To examine whether the insulin-like signaling pathway regulates the expression of genes that encode antioxidant defense enzymes, we measured the level of mRNA transcripts of SODs and catalase in the *age-1* mutant. The level of *sod-3* mRNA in the *age-1* was significantly higher than that in the wild type. The level of mRNA transcripts of *sod-1*, *sod-2* in the *age-1*, was similar to those in the wild type (Figure 3). We also examined

Figure 3: RT-PCR analysis of the level of gene expression of various *sods* and *ctl-1* in *C. elegans* mutants. The mRNA of the ribosomal protein; *rp21c* served as the initial RNA quantitation for each sample.

additional allele of *age-1*. The level of *sod-3* mRNA in the *age-1(hx542)*; *fer-15(b26)* and *age-1(hx546)*; *fer-15(b26)* mutants was higher than that of the *fer-15(b26)* control mutant (data not shown). The elevated level of *sod-3* mRNA in the *age-1* mutant was suppressed by the *daf-16(m26)* mutation and was not fully suppressed by the *daf-18(e1375)* mutation (Figure 3). The level of *ctl-1* *yom* mRNA in the *age-1* was higher than that in the wild type as previously shown by Taub *et al.* (39). The elevated level of *ctl-1* mRNA in the *age-1* mutant was suppressed the *daf-16(m26)* mutation. The level of *ctl-1* mRNA in the double mutant of *age-1(m333)* and *daf-18(e1375)* was higher than that of the *daf-18(e1375)* mutant, indicating that *daf-18* mutation did not fully suppress the increased expression of *ctl-1* gene in the *age-1* mutant. This provides further evidence that a correlation exists between the Age and Oxr phenotypes and expression of *sod-3* and *ctl-1* in the insulin-signaling pathway.

Life-span extension by short-term exposure to hyperoxia

The wild type strain that was reared under normoxic condition, was exposed to 90% oxygen for 2 days from a 6-day adult age. The life span was measured after it was returned to normoxic condition until the end of life. Figure 4 showed slight, but nevertheless significant, increases in mean and maximum life span after 2-day exposure to 90% oxygen. The control animals were reared under normoxic conditions throughout life.

Gompertz analysis of aging of hyperoxia-exposed animals

It is well known that one of the best criteria of the aging process is an exponential acceleration of mortality rate with chronological age, which is formulated by the Gompertz equation (see Materials and Methods). The acceleration rate, i.e., the Gompertz component α , is a parameter of aging rate and has been estimated in *C. elegans* (40). As shown in Table 1, the Gompertz analysis of the survival data of control and hyperoxia-

exposed animals was performed by three different ways as mentioned in the footnote of Table 1. In any analyses, the Gompertz component α of the hyperoxia-exposed animals was shown to be smaller than that of the control animals, indicating that short-term exposure to hyperoxia slowed the aging rate.

Table 1. Life span extension by hyperoxia

	Mean life span±SE (days)	Max. life span (days)	Gompertz parameters (day ⁻¹)						n
			Method 1		Method 2		Method 3		
			α	M ₀	α	M ₀	α	M ₀	
air	17.3 ± 0.35*	34	0.094	0.063	0.080	0.031	0.229	0.003	215
90% oxygen	18.6 ± 0.46*	36	0.088	0.049	0.073	0.028	0.193	0.004	162

Gompertz parameters were calculated by linear regression (Method 1) and non-linear regression (Method 2) analysis of Gompertz mortality function, and non-linear regression analysis of Gompertz survival function as described by Wilson (50) (Method 3) using the Kaleida Graph program (Synergy Software).

* $p = 0.018$. Survival data were analyzed by Kaplan-Meier estimation followed by the log-rank test.

Figure 4: Life span extension by 2-day exposure to hyperoxia.

Adaptive response to oxidative stress

To test whether hyperoxia exposure can increase lethal oxidative stress resistance, the wild type strain was pre-exposed to 90% oxygen for 2 days, and subsequently treated with 50 mM PQ under 98% oxygen, compared to animals which was treated with 50 mM PQ under 98% oxygen without pre-exposure. As shown in Figure 5, exposure to 90% oxygen increased oxidative stress resistance. To examine how long the increased oxidative stress resistance induced by hyperoxia can persist, we measured time course of oxidative stress resistance as a function of time period after returning from 90% oxygen exposure to normoxic oxygen. As shown in Figure 5, after exposure to 90% oxygen, oxidative stress resistance decreased gradually. Seven days after 90% oxygen exposure, animals showed similar oxidative stress sensitivity to untreated animals. This indicated that exposure to 90% oxygen induced an adaptive response for protection against oxidative stress supporting the observation of Darr and Fridovich (37).

Figure 5: Effect of intervals between preexposure to 90% oxygen and PQ treatment on PQ toxicity. At various period after pre-exposure to 90% oxygen, animals were treated with PQ 50 mM under 98% oxygen. The number of animals: no pre-exposure to 90% oxygen; 224, 0 day interval; 47, 1 day; 46, 3 days; 55, 5 days; 38, 7 days; 75.

Induction of gene expression of antioxidant enzymes by hyperoxia

To test whether hyperoxia can induce the gene expression of antioxidant enzymes, we measured the gene expression of 3 types of SODs and catalase after exposure to 90% oxygen. Figure 6 showed the time course of induction of the gene expression of *sod-1*, *sod-2*, *sod-3* and catalase after 90% oxygen exposure indicating induction of various antioxidant enzyme genes.

Figure 6: Northern blot analysis of various SOD and catalase genes in the wild type under 90% oxygen. The mRNA of the ribosomal protein; *rp21c*, serve as the RNA quantitation reference for each sample.

DISCUSSION

Oxidative stress resistance and expression of antioxidant enzymes in life span extension mutants

We showed that the life span extension *age-1* mutants were resistant to PQ, an O_2 -dependent intracellular O_2^- generator under hyperoxia (98% oxygen) confirming that *age-1* mutation confers oxidative stress resistance (30, 41). We found that Oxr phenotype of *age-1* mutant was suppressed by a *daf-16* mutation

indicating that *daf-16* locates downstream of *age-1* in the pathway for regulating Oxr. On the other hand, we found that Oxr phenotype of *age-1* mutant was not fully suppressed by a *daf-18(e1375)* mutation, indicating that *daf-18* does not act downstream of *age-1* in the Oxr pathway. Previously we found that *daf-16* and *daf-18* act downstream of *daf-2* in the insulin-like signaling pathway for Oxr (29). Taken together, we postulate a following pathway for Oxr:

daf-2 → *daf-18* → *age-1* → *daf-16* → Oxr

This pathway is essentially the same as the pathway for regulating longevity (11), suggesting a strong association of life span extension and oxidative stress resistance. Dorman & Canyon demonstrated that the *daf-18(e1375)* mutation suppressed Age phenotype of other allele of *age-1(hx546)* so that *daf-18* acts downstream of *age-1* (16). Such differences could be attributed to the differences in the severity of the *age-1* alleles being used.

age-1 encodes PI3 kinase which phosphorylate PIP_2 to generate the second messenger PIP_3 (Figure 7). The second messenger has been suggested to activate Akt-family kinases, AKT-1 and AKT-2, the *C. elegans* homologues of the mammalian Akt/PKB protooncogene (20) and finally antagonize the activity of DAF-16 transcription factor. PTEN encoded by *daf-18*, dephosphorylates PIP_3 , and thereby degrades this second messenger (42).

Figure 7: Model for regulation of longevity by insulin-like signaling.

In the wild type, a PIP_3 level maintained under a balance between the generation by PI3 kinase and the degradation by PTEN could inhibit the DAF-16 transcription factor activity. The loss or reduction of function mutation in *age-1* could reduce PI3 kinase activity to drop this second messenger level to finally activate DAF-16. In the double mutant of *age-1* and *daf-18*, only preexisting pool of the second messenger could not fully inhibit DAF-16 activity.

Since *daf-16* encodes an HNF-3 / Fork head family transcription factor (9, 10), it can be considered that the *age-1* mutation activates the transcription of a set of genes involved in oxidative stress resistance. Some of

these genes should be component(s) of antioxidant defense systems. Interestingly, we found that the *age-1* mutants have an elevated level of mRNA transcripts of *sod-3*, which encodes Mn-SOD located in the mitochondria, a major site of O_2^- generation. We also found that the *daf-16* mutation suppressed both the Oxr phenotype and the increased level of *sod-3* mRNA in the *age-1* mutant and that the *daf-18* mutation did not fully suppress these phenotypes. Previously we found that *daf-2* mutations upregulate *sod-3* gene expression and that *daf-16* and *daf-18* mutations suppress it. These results suggest that the insulin-like signaling pathway for life span extension also confers oxidative stress resistance by regulating the Mn-SOD gene expression. This strongly suggests that life span extensions by these mutations are caused by efficiently withdrawing ROS that are generated during normal metabolism.

Life span extension by short-term exposure to hyperoxia

Exposure of *C. elegans* to hyperoxia adaptably increases resistance to subsequent lethal oxidative stress for several days and increases expression of antioxidant defense enzymes such as SOD (Fig. 5 and 6). These adaptive animals seem somewhat like the life span extension mutants such as *age-1* or *daf-2*, in that these mutants display Oxr phenotype and upregulation of the Mn-SOD. This notion led us to examine the life span of animals after short-term exposure to hyperoxia. We showed that exposure to hyperoxia for 2 days slightly but significantly extended the mean and maximum life span. The most plausible explanation is that an increase in ability to withdraw ROS induced by hyperoxia could reduce normally occurred oxidative stress that could be the cause of aging.

There is accumulating evidence that suggests the involvement of oxidative stress in the aging process of *C. elegans*. In a previous study on *C. elegans*, we reported that the life span was extended under hypoxia and was inversely correlated with the concentration of environmental oxygen (3, 36). The oxidative stress sensitive *mev-1*, a homologue of succinate dehydrogenase cytochrome b, mutant has a shorter life span, and a faster aging rate, than the wild type (3, 43). Hartman *et al.* also demonstrated an inverse correlation between life span and susceptibility to oxidative stress, in recombinant inbred strains (44). Cytosolic catalase was reported to be required for the life span extension of *age-1*, *daf-2* and *clk-1* mutants (39). The addition of SOD / catalase mimetics into medium were shown to extend the life span of *C. elegans* (45). These findings suggest that oxidative damage is an important causative factor in the aging process of *C. elegans*. Taken together, the close correlation between life span extension and oxidative stress resistance and increased gene expression of antioxidant enzymes as shown in the present study, suggests that life span could be extended by a slowing in the rate of accumulation of oxidative damage caused by normal metabolism.

EXPERIMENTAL PROCEDURES

Strains and Culture of Nematodes

The hermaphrodite *C. elegans* strains were maintained at 2°C on NG agar medium with *Escherichia coli* OP50 as a food source, as described by Brenner (46). The N2 Bristol strain was used as the wild type.

The strains used in this study were:

Linkage group LG I: *daf-16(m26)*

LG II: *fer-15(b26ts)*, *age-1(hx542)*; *fer-15(b26ts)*, *age-1(hx546)*; *fer-15(b26ts)*, *age-1(m333)/mnC1 dpy-10(e128) unc-52(e444)*, *age-1(mg44)/mnC1 dpy-10(e128) unc-52(e444)* LG IV: *daf-18(e1375)*

Life span

Eggs were allowed to hatch for 2 hours. Larvae that hatched during this period were transferred onto a fresh plate and monitored daily until death. The first day of adulthood is day 0 in the survival curves presented. Animals that did not move after gentle mechanical touch were scored as dead. Animals that had died by hatching of progeny inside the uterus were not counted.

Oxygen exposure

The animals in the plate were exposed to 90% oxygen in an airtight plastic chamber as described previously (3). The chamber was opened every day, and the gas (90% oxygen) was replaced.

Gompertz analysis

The Gompertz analysis of the survival data of nematodes was performed using the Gompertz equation as follows:

$$M_t = D_{\Delta t} / \Delta t L_t = M_0 e^{\alpha t}$$

Where M_t represents mortality rate, t chronological age, $D_{\Delta t}$ the number of animals dying between age t and age t plus Δt , L_t the number of animals alive at the age t , M_0 initial mortality rate (mortality rate at birth), and α the Gompertz component (the acceleration rate of age-specific mortality) (47). Gompertz parameters α and M_0 were calculated by three ways as described in the footnote of Table 1.

Oxidative stress resistance assay

Approximately twenty hermaphrodites of *C. elegans* at the L4 stage, were placed in a solution which consisted of 50 mM PQ (Sigma), 0.2 ml S medium, and OP50 1×10^7 /ml, in 15-mm diameter dishes (4-well Nunclon multiplates; Intermed, Roskilde, Denmark) and then exposed to 98% oxygen as described above. The chamber was opened every twelve hours, at which time the number of surviving animals were counted, and the gas (98% oxygen) was replaced.

Northern blot analysis

Messenger RNA (mRNA) was isolated using the guanidinium-acid-phenol-chloroform method (48), and Oligo (dT)-Cellulose Spun Columns (Amersham Pharmacia Biotech, Uppsala, Sweden), from *C. elegans* at the young adult stage. Two micrograms of mRNA was separated on 1.2% agarose-formaldehyde gels, and blotted onto a nylon membrane (Magna Graph, MSI,

MA). The relative amount of mRNA was judged by hybridization with a *C. elegans* ribosomal protein, *rp21c* cDNA probe (Eco RI/Sma I fragment of pPD33.24). The *C. elegans* *sod-2* cDNA probe was the Kpn- I/Sac -I fragment of BTK65-1 (32). The *C. elegans* catalase cDNA probe was the Eco RI fragment of a CeCAT (X82175) clone. The cDNA probes of *C. elegans* *sod-1* and *sod-3* cDNA were synthesized by reverse transcription-polymerase chain reaction (RT-PCR) (Titan One Tube RT-PCR System, Boehringer Mannheim, Mannheim, Germany), from total RNA of *C. elegans*; the oligo DNA primers used for *sod-1* were 5'-AGGTCGAAGC CGCTCAAAAAA-3' and 5'-ATTGTGTGGAGATTCAGAGA, and those used for *sod-3* were 5'-AATGCTGCAATCTACTGCTC-3' and 5'-AGCGTTTTAACTACATCTG-3'. The cDNA was amplified (35 cycles; 94°C, 1min; 55°C, 1min; 72°C, 1min) and the PCR products were subcloned into the pGEM-T vector (Promega, Madison, WI) for amplification. Hybridization was carried out in a solution which consisted of 5 x SSPE, 10 x Denhardt's reagent, 50% formamide, 14% SDS, and 0.1mg/ml herring sperm DNA with ³²P-labeled probes at 42°C (49). After hybridization, the membrane was washed and the degree of hybridization was estimated by using a Fujix BAS 2500 Laser Image Analyzer (Fuji Film, Kanagawa, Japan).

RT-PCR analysis

Total RNA was isolated by the guanidinium-acid-phenol-chloroform method (48). For RT-PCR, 1-100 pg RNA was used as the template. The oligo DNA primers used in the Northern blot analysis of *sod-1* and *sod-3* were used for the RT-PCR. The oligo DNA primers used to detect PCR product were as follows: *sod-2* (5'-CTTCAAAACACCGTTTCGCTG-3' and 5'-CAGTGGAAACAAGTCCAGTT-3'); *rp21c* (5'-GCTTGCGTCTACCTGCTC-3' and 5'-TCCGGAAGAGACAGAAGTGA-3'). The primer sets used for the amplification of *sod-2* did not amplify the cDNA of *sod-3*, and the primer sets used for *sod-3* did not amplify the cDNA of *sod-2*. The PCR products were amplified with the following amplification profile for 35 cycles: denaturation for 1min at 94°C, annealing for 1min at 55°C and extension for 1min at 72°C.

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Welfare of Japan.

ABBREVIATIONS

Age - life span extension
Oxr - oxidative stress resistance
PDK1 - phosphoinositide-dependent kinase-1
PI3 kinase - phosphoinositide 3-kinase
PIP₃ - phosphatidylinositol-3,4,5-triphosphate
PIP₂ - phosphatidylinositol-4,5-bisphosphate
PQ - paraquat, 1,1'-dimethyl-4,4'-bipyridinium dichloride
ROS - reactive oxygen species
SOD - superoxide dismutase
O₂^{·-} - superoxide radical

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