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Unincorporated iron pool is linked to oxidative stress and iron levels in *Caenorhabditis elegans*

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Abstract Free radicals or reactive oxygen species (ROS) are relatively short-lived and are difficult to measure directly; so indirect methods have been explored for measuring these transient species. One technique that has been developed using *Escherichia coli* and *Saccharomyces cerevisiae* systems, relies on a connection between elevated superoxide levels and the build-up of a high-spin form of iron (Fe(III)) that is detectable by electron paramagnetic resonance (EPR) spectroscopy at $g = 4.3$. This form of iron is referred to as "free" iron. EPR signals at $g = 4.3$ are commonly encountered in biological samples owing to mononuclear high-spin ($S = 5/2$) Fe(III) ions in sites of low symmetry. Unincorporated iron in this study refers to this high-spin Fe(III) that is captured by desferrioxamine which is detected by EPR at g value of 4.3. Previously, we published an adaptation of Fe(III) EPR methodology that was developed for *Caenorhabditis elegans*, a multi-cellular organism. In the current study, we have systematically characterized various factors that modulate this unincorporated iron pool. Our results demonstrate that the unincorporated iron as monitored by Fe(III) EPR at $g = 4.3$ increased under conditions that were known to elevate steady-state ROS levels in vivo, including: paraquat treatment, hydrogen peroxide exposure, heat shock treatment, or exposure to higher growth temperature. Besides the exogenous inducers of oxidative stress, physiological aging, which is associated with elevated ROS and ROS-mediated macromolecular damage, also caused a build-up of this iron. In addition, increased iron availability increased the unincorporated iron pool as well as generalized oxidative stress. Overall, unincorporated iron increased under conditions of oxidative stress with no change in total iron levels. However, when total iron levels increased in vivo, an increase in both the pool of unincorporated iron and oxidative stress was observed suggesting that the status of the unincorporated iron pool is linked to oxidative stress and iron levels.

Keywords Oxidative stress · Reactive oxygen species · Iron electron paramagnetic resonance · Free iron · Unincorporated iron · Iron

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Introduction

Reactive oxygen species (ROS) play various important roles in living systems. However, over-production of these prooxidants leads to a cellular condition

referred to as oxidative stress, which has been implicated in numerous diseases and aging (Halliwell and Cross 1994; Melov 2000; Halliwell and Gutteridge 1999; Christen 2000). Due to instability of ROS, it is challenging to measure their levels in vivo; as a result, there is a constant search for novel and reliable methods for quantification of these species. In recent years, largely in the unicellular organisms *E. coli* and *S. cerevisiae,* the absence of the superoxide scavenging enzyme, superoxide dismutase (SOD), has been correlated to the hyper-accumulation of a non-heme, high-spin rhombic ferric iron species that is detectable by low temperature Fe(III) Electron Paramagnetic Resonance (EPR) at $g = 4.3$ which has been referred to as "free" iron (Keyer and Imlay 1996; Srinivasan et al. 2000; Srinivasan and Gralla 2002). This EPR-detectable, or the so called "free" iron, was not believed to be newly acquired by the cell since total iron levels did not deviate significantly from the levels displayed by the wild-type yeast (Srinivasan et al. 2000). The build-up of this form of iron was believed to be caused by superoxide stress (due to an overproduction of superoxide anion) as it was alleviated by conditions that reduced superoxide levels, such as expression of a non-endogenous SOD or growth under anaerobic conditions (Srinivasan et al. 2000). In rats, elevated EPR-detectable Fe(III) levels at $g = 4.3$ correlated with elevated lipid peroxidation measured by thiobarbituric acid test, indicating the link between oxidative stress and EPR-detectable Fe(III); interestingly, the effect was also observed in rats fed an iron supplemented diet (Kozlov et al. 1996). This coincided with what was seen in the *E. coli sod* mutants as this signal intensified further when the iron regulon (Fur) was disrupted (Keyer and Imlay 1996) suggesting a link between iron availability and "free" iron as measured by EPR as well.

In biological samples EPR signals at $g = 4.3$ are commonly encountered owing to mononuclear high-spin ($S = 5/2$) Fe^{3+} ions in sites of low symmetry. Although we and others have used the terminology "free" iron or adventitious iron to refer to this form of iron in the past, to be consistent with more recently published work in *E. coli* (Varghese et al. 2007; Liu et al. 2011), in this study we will use the terminology unincorporated iron to refer to the iron pool that can be captured by desferrioxamine to yield an Fe(III) EPR signal at $g = 4.3$. This EPR signal measures high-spin rhombic Fe(III) that is not bound to heme-containing proteins or Fe–S cluster proteins, as the iron bound to these types of proteins produce EPR signal at distinct g values. Clearly, there is no such chemical entity as free iron in an intracellular environment as it would undoubtedly exist only in complexes with other cellular components. The term unincorporated or "free" iron denotes a loosely bound form of iron that prior to capture by desferrioxamine has an unknown coordination environment and may not represent a single species or compound. We have previously compared samples of the nematode *Caenorhabditis elegans* that were prepared with and without the addition of desferrioxamine (Pate et al. 2006), and the signal at $g = 4.3$ increased more than 2-fold with desferrioxamine treatment. This result indicates that the pool of iron being measured in *C. elegans* contains unincorporated ferrous iron that was oxidized to EPR-detectable ferric iron upon the addition of desferrioxamine. Since we have compared the spectra of samples with and without desferrioxamine, we can define at least one component of the unincorporated iron measured here as the iron that can be captured by desferrioxamine, which is detected at $g = 4.3$. We expect this pool to include iron that is loosely bound to the surfaces of proteins, DNA, and lipids.

The recent discovery that yeast null for *SOD1* cultured in minimal medium for as few as 24 h, as opposed to the previously reported 72 h-vegetative growth studies, also exhibit a 2–3-fold increase in EPR-detectable iron at $g = 4.3$ provide further evidence for a superoxide stress-induced effect (Clement, Valentine, unpublished). Several lines of evidence now indicate that the EPR-detectable iron at $g = 4.3$ is likely localized to the mitochondrion in yeast. Although approximately 60 % of mitochondrial iron is in 4Fe–4S clusters (Hudder et al. 2007), the mitochondrial "adventitious iron" pool, which makes up about 22 % of mitochondrial iron, is composed of both high-spin non-heme ferrous and ferric iron that is detectable by EPR at $g = 4.3$ (Hudder et al. 2007). A study in *E. coli* demonstrated that very low levels of H_2O_2 are also capable of damaging the enzymatic activity of 4Fe–4S cluster containing proteins similar to superoxide radicals (Jang and Imlay 2007). Additionally, H_2O_2 although to a limited extent can diffuse across membranes (Seaver and Imlay 2001) enabling it to access the "adventitious iron" pool of the mitochondrial intermembrane space (IMS) and

potentially oxidizing it to a high-spin ferric form that is detectable by EPR. Based on this evidence, it is plausible that this is not a superoxide specific effect and that other ROS, including hydrogen peroxide, may contribute to the unincorporated iron pool.

In our previous study we successfully extended the Fe(III) EPR methodology to a multi-cellular organism, *C. elegans*, with the eventual goal of correlating life-span and oxidative stress tolerance with ROS levels (Pate et al. 2006). The idea was that this EPR-based method could report oxidative stress status in vivo via the quantification of organismal unincorporated iron levels. Similar to the *E. coli* and yeast system, whole worms also produced an Fe(III) EPR signal at $g = 4.3$ (Pate et al. 2006). Following the procedure used with *E. coli*, a cell-permeable chelator, desferrioxamine, was used in *C. elegans* sample preparation to oxidize a portion of this pool that was present in Fe(II) form prior to measurement using Fe(III) EPR. The goal of the initial study in *C. elegans* was for the purpose of methodology development and for the validation of various steps introduced in sample preparation; some preliminary determinations were then made to see if there was a correlation between Fe(III) EPR signal at $g = 4.3$ and oxidative stress. Conditions that were known to alter in vivo ROS levels, such as heat shock and paraquat exposure, modulated the EPR signal either positively or negatively warranting further detailed investigation.

In the present study, we set out to systematically characterize various factors that alter the EPR-detectable Fe(III) at $g = 4.3$ (unincorporated iron pool) to learn if the build-up of this form of iron was exclusively mediated by superoxide or if any ROS result in the effect. Our results demonstrate that the unincorporated iron signal responded to any treatment that is known to enhance steady-state ROS level, suggesting that besides superoxide, other ROS such as hydrogen peroxide can also raise this pool of iron. In addition to external inducers of oxidative stress, physiological aging also caused the unincorporated iron signal to increase with no change detected in total iron. Iron-replete growth medium which caused a boost in total iron in vivo produced the largest increase in the Fe(III) EPR signal. This was accompanied by an increase in oxidative stress. Collectively, these findings provide a link between cellular iron (total and unincorporated) levels and in vivo oxidative stress status.

Materials and methods

Materials

Caenorhabditis elegans wild-type, N2 and TJ1060 [*spe-9* (*hc88*) *I; fer-15* (*b26*) *II*], and TK22 (*mev-1*[*kn1*] *III*) strains were obtained from the *C. elegans* Genetic Stock Center. The *E. coli* OP50 strain was obtained from Dr. Catherine Clarke's Laboratory (UCLA). *C. elegans* cultivation media supplies were purchased from Fisher Scientific and deferoxamine mesylate (commonly known as desferrioxamine), methyl viologen (paraquat) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Sigma-Aldrich. Quartz EPR tubes were purchased from Wilmad Glass. Buffers used include: M9 (200 ml 5× M9 Salts, 1 ml 1 M $MgSO_4$, and 799 ml nanopure water per liter of M9 buffer), 5× M9 Salts (30 g Na_2HPO_4, 15 g KH_2PO_4, 5 g NH_4Cl, and 2.5 g NaCl per liter of solution), 15 % glycerol buffer (15 % v/v diluted in M9), and alkaline hypochlorite solution (10 ml nanopure water, 1 ml 5 M NaOH, and 0.8 ml commercial bleach).

Concentrated *Escherichia coli* (OP50) preparation

One milliliter of seed OP50 culture was added to 1 l of LB broth and allowed to grow at 37 °C in a shaker incubator for 15–18 h. The culture was centrifuged for 10 min at 4 °C and 4,000 rpm, and the pellet was resuspended in 7 ml of M9. This concentrated OP50 was aliquoted and stored at −20 °C until needed for seeding of the NGM agar plates.

Caenorhabditis elegans cultivation and synchronization

Worms were grown on the nematode growth medium (NGM) agar plates according to standard protocol (Hope 1999). In specific cases where iron supplementation of the NGM was desired, ferric ammonium citrate (FAC) was supplemented to the NGM agar dry mixture (for 25 mM, 6.6 g of FAC per L of NGM agar). The solidified agar plates were seeded with OP50, a strain of *E. coli* used as a food source, and worms were transferred to a new plate by moving an agar chunk containing worms from a stock plate. After several days at 22 °C, standard growth temperature used in our laboratory, the worms predominantly in

the gravid stage were washed off the plates using sterile M9 solution. The worms were separated from the bacteria using successive centrifugation at 4 °C and washing in M9, and then subjected to an alkaline hypochlorite treatment to isolate the eggs. In the case of worms grown on FAC-treated plates, they were subjected to three additional washes with 1 mM EDTA followed by a final M9 wash prior to isolating the eggs. The collected eggs were placed on new NGM plates containing concentrated OP50 and incubated at 22 °C (for approximately 48 h) and allowed to grow to the larval stage 4 (L4 stage), thus producing a synchronized population.

EPR sample preparation

EPR samples were prepared according to previously published procedure (Pate et al. 2006). Briefly, the synchronized worms at the L4 (or any specified) stage were removed from the plates by washing using M9, counted to obtain worm number, and then prepared for EPR as described earlier using desferrioxamine treatment and a brief homogenization. The number of worms used in each EPR sample ranged from 12,000 to 25,000. However, within a given experiment the same number of worms was used for both the control and the experiment. Once the samples were transferred to clean quartz EPR tubes, they were flash frozen in a dry ice/ethanol bath immediately and stored at −20 °C until EPR measurements were performed.

Short-term exposure to hydrogen peroxide

Hydrogen peroxide was added to the washed and counted L4 worms in M9 resulting in a final H_2O_2 concentration of 2 mM. To the control sample, vehicle (water) was added. Both samples were allowed to incubate on a tilting platform for 30 min at room temperature; the worms were collected and washed four times in M9. Both samples were then prepared for EPR analysis following the EPR sample preparation protocol mentioned above.

Short-term exposure to varied temperature

Eggs on NGM agar plates were developed to L3 stage at 22 °C (typically for 36 h after egg isolation) and then subjected to varied temperature exposure for 12 h by moving the worms to either a 15, 22, or 25 °C incubator. After 12 h, all bacteria were then removed as usual by repeated washing with M9 and the standard EPR sample preparation methodology was followed to obtain samples for the EPR analysis.

Heat shock and paraquat exposure

For heat shock exposure, L4 worms were placed in a 35 °C incubator for the required time period, while control samples were maintained for the same amount of time at 22 °C. For paraquat treatment, L4 staged worms were exposed to the indicated levels of paraquat in solution for 1 h at 22 °C with rocking, while the control was treated with vehicle (water). EPR sample preparation was carried out as described previously after removing bacteria using successive washes.

Preparation of aged worms

The temperature-sensitive wild-type strain, TJ1060, was used to obtain a continuous stream of synchronized population for the aging experiment. These hermaphrodites reproduce at 15 °C, but are sterile when shifted to 25 °C. Hence, to allow growth and fertilization, these worms were maintained at 15 °C and after the isolation of eggs from gravid worms, eggs were incubated at 25 °C. One EPR sample was prepared when the worms reached the L4 stage (approximately 48 h after the egg isolation) and another sample was prepared five days after the L4 EPR sample withdrawal using the same number of worms for both EPR samples.

Low temperature Fe(III) EPR at $g = 4.3$

EPR spectra were recorded using a Bruker X-band spectrometer located at the University of California Los Angeles (Los Angeles, CA) at a g-value of 4.3. EPR measurements were carried out at 98 K using previously published parameters (Pate et al. 2006). Parameters used for low-temperature Fe(III) EPR: center field: 1,560 G, sweep width: 500 G, microwave power: 31.8 mW, attenuation: 8 dB, modulation amplitude: 20.0 G, modulation frequency: 100 kHz, receiver gain: 2×10^5, sweep time: 20.97 s, time constant: 81.92 ms, conversion time: 10.24 ms, resolution: 2,048 points, number of scans: 8. In all of the spectra the background signal from the empty cavity

was subtracted out. EPR data processing was done using Bruker WinEPR software and unincorporated iron quantitation was done by double integrating the signal obtained after baseline correction using the software. Each day, the spectrum of a iron(III) desferrioxamine standard (22 μM) was recorded under identical conditions as that of samples and the double integral value of the standard was compared with the sample to determine their unincorporated iron concentrations. In all our samples relative comparisons were made and the desferrioxamine-bound iron at $g = 4.3$ was quantified by double-integration. Desferrioxamine creates the iron coordination environment, which was always identical in all of our samples since all samples in this study contained desferrioxamine. Because the zero-field splittings due to desferrioxamine are relatively small ($D = 1.2$ cm^{-1}) and all spectra are recorded at 98 K it is safe to assume that the equal double integrals of the Kramer's doublets are representative of their equal relative populations. Therefore, it is appropriate to take the double integral value of the peak at $g = 4.3$ for Fe(III) captured by desferrioxamine as an indicator of the concentration or population of that state. The unincorporated iron levels reported reflects the concentration of unincorporated iron in a discrete population of worms, in the form of a homogenate, and it is not the concentration on an intraworm basis. To avoid averaging of data collected on worms grown on various days, the unincorporated iron fold increase was used rather than the unincorporated iron concentration. Typically, each sample was prepared in duplicate (or more) on a given day and the growth and sample preparation was repeated 2–5 independent times. EPR spectra shown in figures were recreated using SigmaPlot after subtracting the background signal and by averaging the spectra for each condition collected on the same day.

Total iron measurements using ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) measurements were carried out as per the published method (Lin et al. 2006). In brief, worm homogenates (from about 1,000 worms) were collected, at least in duplicates, after EPR measurements and digested in 1 ml of 20 % nitric acid (OPTIMA Nitric Acid, Fisher Scientific) at 98 °C for 18 h. The digested samples were diluted to 2.7 ml in nanopure water and iron levels were measured using a Hewlett Packard (HP)-4500 ICP-MS at California Institute of Technology (Pasadena, CA). A standard curve was generated using iron standards of known concentration (10–200 ppb) and using this curve total iron levels in the unknowns were determined.

Oxidative stress measurements using DCFDA

2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used as a molecular probe to detect intracellular ROS in *C. elegans*. The wild-type (N2) worms were grown under specified conditions from eggs for 2 days. Worms were picked off the plate and then incubated in 10 μM 2′, 7′-dichlorodihydrofluorescein diacetate solution for 30 min. Worms were then fixed on 2 % agarose pads containing 15 mM sodium azide on microscopic slides. In each individual experiment, images of 10 worms per condition were captured under the Leica TCS SP2 laser scanning confocal microscope set at an excitation wavelength of 488 nm and an emission wavelength window between 498–522 nm. Representative images presented are chosen by identifying the pattern displayed by majority of the worms (greater than 90 % of the worms examined) under a given condition. Each experiment was repeated three or four independent times.

Data representation and statistical analysis

In all the figures presented, average plus or minus standard deviation is shown. Statistical calculations were performed using MINITAB® Release 14 Statistical Software. For any given EPR experiment, samples for control and experimental were prepared from growth, in duplicate (or more), on many separate days. Therefore, to determine the statistical difference between conditions, the effects of both the day prepared and the variable treatment were considered. First, a two-way ANOVA was performed to determine the contribution of separate preparation days to the variability among the unincorporated iron levels. If the separate days did contribute, the variability due to the specific treatment condition was determined based on an F test from the two-way ANOVA. If the separate days did not contribute, the variability due to the specific treatment condition was determined based on an F test from a one-way ANOVA. P value less than 0.050 were considered to be statistically significant.

Results

In unicellular organisms "free" iron as measured by the low-temperature Fe(III) EPR at $g = 4.3$ provided an indirect measure of in vivo superoxide levels. Earlier, we developed this methodology for *C. elegans* and after working out the multi-step protocol we showed that (i) *C. elegans* also show an increase in steady-state level of this form of iron that is detectable by EPR in the presence of desferrioxamine and (ii) the Fe(III) EPR signal at $g = 4.3$ was sensitive to superoxide and heat stress that is documented to overproduce ROS. In the present study we have systematically characterized the factors that modulate Fe(III) EPR signal observed in the presence of desferrioxamine at $g = 4.3$ to explore if it can provide an indirect read-out of oxidative stress status in *C. elegans*. We monitored the unincorporated iron signal response to various treatments, such as exposure to iron, paraquat, hydrogen peroxide, and heat shock that alter either the cellular iron status or ROS.

Paraquat treatment induces varying responses that are condition dependent

Paraquat (1,1′dimethyl-4-4′-bipyridynium dichloride), is a commonly used herbicide and its toxicity in vivo is mainly due to the generation of the superoxide anion (Halliwell and Gutteridge 1999; Cocheme and Murphy 2008), which can also lead to the production of other ROS (Smith et al. 1978; Hassan and Fridovich 1978). Fe(III) EPR signal increases have previously been shown to increase in wild-type *E. coli* and yeast exposed to paraquat (Srinivasan et al. 2000; Keyer and Imlay 1996) presumably due to an analogous build-up of superoxide and/or the synthesis of other ROS that occurs in superoxide dismutase knockouts; in an attempt to learn more about the effect of ROS on this iron pool in *C. elegans,* we chose to investigate the influence of paraquat.

Collectively, our results (past and current), demonstrate that depending on the concentration of paraquat used, exposure time, and the growth stage of the worms, notably different trends are observed (Table 1). N2 worms, when grown from eggs to the L4 stage (approximately 48 h) on NGM plates containing 1 mM paraquat, displayed a decreased unincorporated iron signal. Exposure of L4 worms, both wild-type and short-lived, superoxide overproducing, *mev-1* (Senoo-Matsuda et al. 2001), to 100 mM paraquat for 1 h resulted in no significant alteration in the unincorporated iron levels. However, exposure to 1 mM paraquat for 2.5 h amplified the observed unincorporated iron signal of the wild-type worms considerably. We speculate that 1 h exposure under our experimental condition is probably not optimal for paraquat uptake and to increase ROS generation registered by this technique. These results indicate that the EPR signal responds to paraquat stress positively, provided the concentration and exposure time are optimal. Samples that showed an alteration in unincorporated iron levels had no change in total cellular iron (data not shown).

Table 1 The effect of varying levels of exposure to paraquat (at 22 °C) and heat shock (at 35 °C) on the measured unincorporated iron in the wild-type *C. elegans*

Stress	Paraquat (mM)	Exposure time (h)	Exposure temperature (°C)	Exposure stage	Unincorporated iron levels[a]	References
Superoxide anion	1	48	22	Eggs—L4	Decrease ($P = 0.019$)	Pate et al. (2006)
	1	1	22	L4	NSC[b]	Pate et al. (2006) and this study
	100	1	22	L4	NSC[b]	This study
	1	2.5	22	L4	Increase ($P = 0.048$)	Pate et al. (2006)
General ROS	0	1.5	35	L4	Decrease ($P = 0.002$)	Pate et al. (2006)
	0	4	35	L4	Increase[b] ($P = 0.024$)	This study

[a] Reported changes in unincorporated iron levels are in comparison to the untreated wild-type (N2) control

[b] For each condition, samples were prepared in duplicate and the experiment was repeated two to four independent times. NSC denotes no significant change

Hydrogen peroxide increases unincorporated iron

Unincorporated iron levels are modulated by paraquat treatment, which has specifically been characterized to increase superoxide levels in vivo. Since superoxide can self-disproportionate to hydrogen peroxide, it is logical to investigate whether the EPR signal can change with direct hydrogen peroxide treatment as well. Hydrogen peroxide is widely used to induce oxidative stress even though it has limited membrane permeability. As with paraquat, L4 worms were exposed to hydrogen peroxide (2 mM) while the control samples were left untreated. A short-term 30-min exposure to this relatively high concentration of hydrogen peroxide was chosen to prevent death. Based on the spectra presented in Fig. 1, there was a greater than 2-fold increase (observed fold increase varied from 1.91 to 2.61 for samples prepared on four independent days; $P = 0.047$) in Fe(III) EPR signal at $g = 4.3$, suggesting that this signal responds positively to hydrogen peroxide.

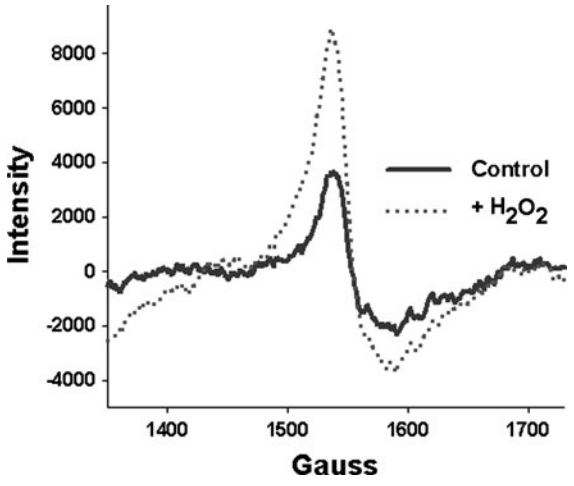

Fig. 1 Short-term exposures to hydrogen peroxide increases the Fe(III) EPR signal. A representative low-temperature Fe(III) EPR spectrum of N2 *C. elegans* without (control) and with exposure to hydrogen peroxide (2 mM) for 30 min is shown. Samples were prepared using the standard EPR sample preparation methodology as described in "Materials and Methods" section. The unincorporated iron concentrations was calculated by obtaining double integration values and calculated based on comparison with a 22 μM iron standard. Observed fold increase in unincorporated iron for H_2O_2 treatment varied from 1.91 to 2.61 for samples prepared on four independent days ($P = 0.047$; $n = 4$)

Unincorporated iron levels are modulated differently with varying levels of heat shock exposure

Unlike paraquat which has been characterized to produce superoxide anions specifically, short-term 35 °C heat shock increases ROS production as demonstrated using isolated mitochondria (Heise et al. 2003). As a result of this stress, *C. elegans* exposed to 35 °C die within 20 h as opposed to the 20 or more days when maintained at an ambient temperature of 22 °C. Based on our previous findings, a short exposure of 1.5–2 h at 35 °C resulted in a consistently lower EPR signal as compared to control worms that were maintained in normal 22 °C environment for the same amount of time (Pate et al. 2006). However, this time period could have been too short to induce heat shock response as the worms take over 3 h to adjust to this temperature by expressing heat shock proteins (Link et al. 1999). Therefore, the heat shock treatment was repeated with an exposure time of 4-h (Table 1). This increased unincorporated iron levels and the worms remained viable. Similar to the paraquat response, Fe(III) EPR signal produces varying results and it is heavily dependent on the length of heat shock exposure. Nonetheless, this signal is modulated positively by any ROS in general.

Increase in growth temperature increases unincorporated iron

It is known that *C. elegans* grown below their normal ambient temperature (18–22 °C) live longer, while exposure to higher temperatures results in a shortened lifespan (Van Voorhies and Ward 1999). This increased longevity at lower temperatures is thought to be in part a result of decreased oxygen consumption, which in turn is believed to decrease free radical formation in vivo. Therefore, to study the production of free radicals that may be mediated by temperature alterations and its effect on the Fe(III) EPR signal response, we exposed *C. elegans* to both higher and lower temperatures than normal (Fig. 2). In this experiment, worms were allowed to grow to the L3 stage at their normal temperature (22 °C) and then switched to the test temperature for 12 h only. This was done to minimize altered rate of development at non-standard growth temperatures, as high temperature is known to accelerate development. The results

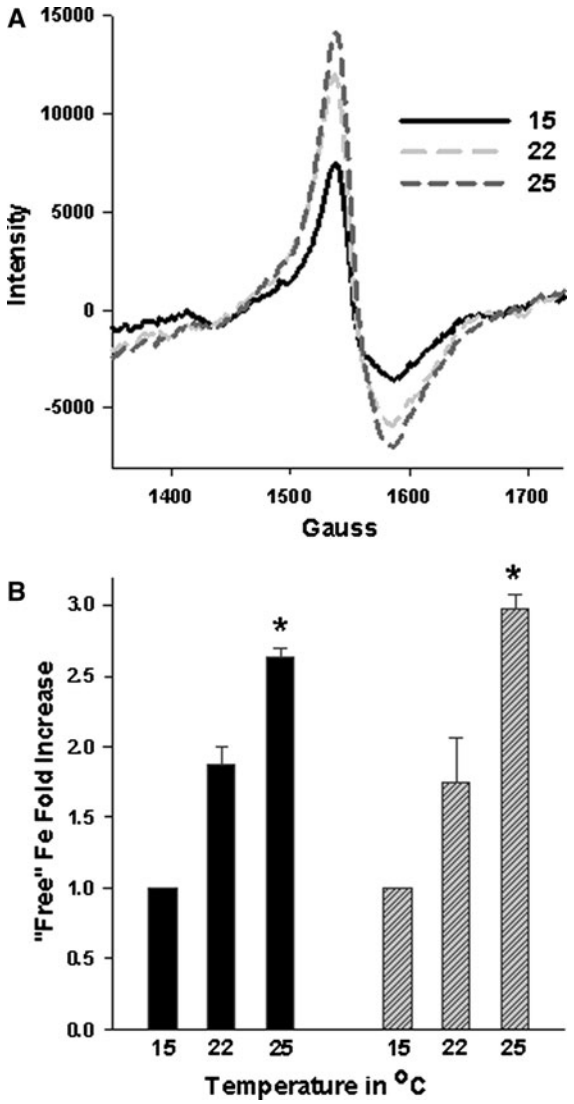

Fig. 2 Growth temperature increase of 10 °C increases unincorporated iron in worms. **A** Low-temperature Fe(III) EPR spectra of N2 *C. elegans* after 12 h exposure to 15, 22 or 25 °C are shown. Two replicates containing the same number of worms were prepared on the same day for each condition and were averaged to obtain the spectra shown. **B** The relative increase of unincorporated iron levels for N2 (*black bars*) and the short-lived *mev-1* (*gray bars*) worm strains are shown. Unincorporated iron levels were normalized to one for the control sample (exposed to 15 °C) for each worm strain; the relative increase in unincorporated iron corresponding to the increased experimental temperature condition is represented with respect to the control. At least six individual samples were analyzed by preparing them on two to four different days. *Denotes statistically significant from the 15 °C control for a given strain based on a two-way ANOVA (*P* value for 15 vs. 25 °C samples for N2 = 0.025 and for *mev-1* = 0.020)

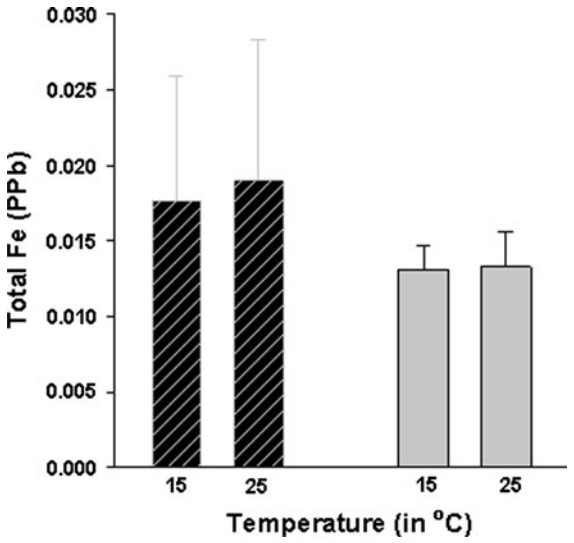

Fig. 3 Total iron levels remain unchanged when grown at an elevated temperature. Total iron levels were measured using ICP-MS from portions of the EPR samples for N2 (*black bars*) and *mev-1* worms (*gray bars*) exposed to 15 or 25 °C as described in the legend for Fig. 2. The values represent the average total iron per worm of two different samples prepared from two different EPR samples

of this experiment show that the unincorporated iron levels were nearly 3-fold higher in both N2 and the short-lived *mev-1* at 25 °C as compared to the worms grown under 15 °C (Fig. 2b). A representative spectrum is shown for the N2 strain in Fig. 2a. ICP-MS measurements were carried out using samples exposed to 15 versus 25 °C to evaluate if the increase in unincorporated iron at higher temperature was due to an increase in total iron accumulation. Within a given strain type, total iron levels were unchanged even when the growth temperature was increased by 10 °C (Fig. 3).

Age effects

Based on the results presented thus far, the unincorporated iron levels increased not only with superoxide stress, but any treatment that has been characterized to overproduce ROS. As a result, we decided to focus on understanding the correlation between unincorporated iron levels with age as oxidative stress and macromolecular damage increases with age. Due to the large number of worms needed for this measurement, a wild-type strain (TJ1060) that is sterile when

cultivated at 25 °C was utilized to maintain an age synchronous population. When comparing L4 worms (2 days post egg hatch typically denoted as day 0 of adulthood) with 5 day old adults, there was an increase in unincorporated iron levels with no significant change in total iron steady-state level (Fig. 4). This suggests that besides treatments that alter ROS concentrations in vivo, physiological aging that is associated with elevated ROS mediated damage, also changes unincorporated iron levels.

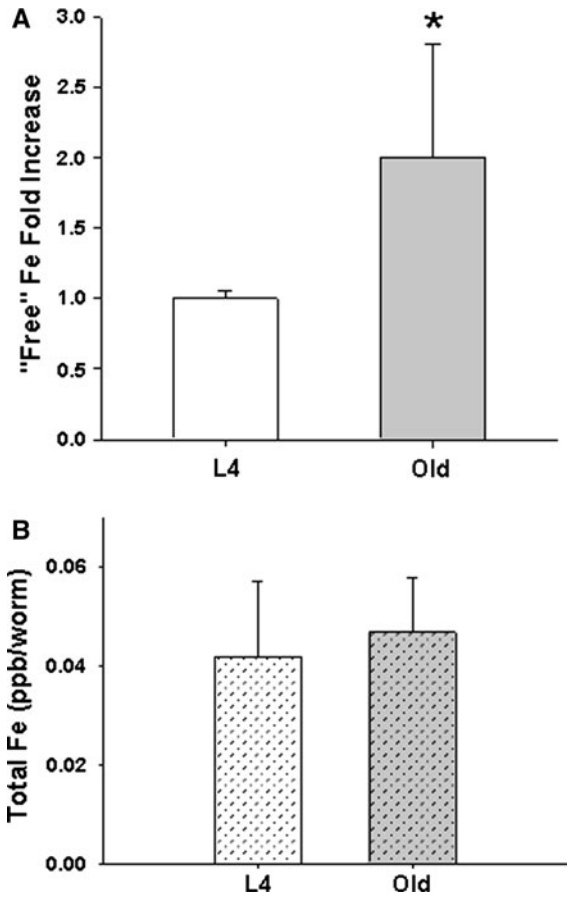

Fig. 4 Unincorporated iron levels are elevated with aging. Wild-type worms (TJ1060 strain) were compared at L4 (day 0 of adulthood) to five days past L4 stage (old). **A** Unincorporated iron levels were normalized to one for the control samples (L4 stage). The relative difference in unincorporated iron levels is representative of six samples prepared from five independent measurements. *Denotes that the relative difference is statistically significant based on a one-way ANOVA ($P = 0.014$). **B** Total iron levels were measured by ICP-MS utilizing aliquots after the EPR measurements. The values represent the average total iron per worm of at least four different samples prepared from two different EPR samples

Iron supplementation causes a dramatic increase in total and unincorporated iron

Since a chemical form of iron that is detectable by EPR is being measured here, it is natural to expect that this signal can change with cellular iron availability. Worms grown on NGM plates supplemented with 25 mM FAC developed normally but exhibited somewhat shorter life spans (Kim et al. 2004). ICP-MS analysis of worms grown under these previously published conditions indicated that 25 mM FAC supplementation leads to the accumulation of significantly more iron in vivo in comparison to controls grown on medium containing normal iron levels. This increase in cellular iron was also accompanied by an enormous increase in unincorporated iron levels (Fig. 5). Besides the 25 mM FAC supplementation, lower concentration of FAC supplementation at 1 mM also produced a greater than 2-fold increase in unincorporated iron levels ($P = 0.017$; $n = 4$; data not shown).

Oxidative stress is induced in worms grown with 25 mM FAC supplementation

In order to assess the in vivo oxidative stress status of worms displaying high levels of total and unincorporated iron due to iron supplementation, DCF

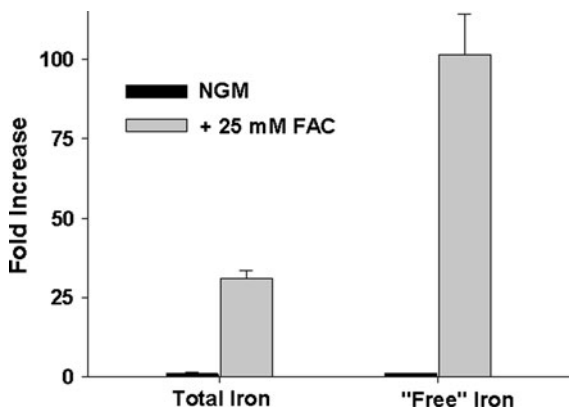

Fig. 5 Iron supplementation increases both the total and unincorporated iron levels in N2. Relative differences in both total iron levels, by ICP-MS, and unincorporated iron levels, by EPR are shown. The EPR data are representative of two samples prepared on same day. ICP-MS measured total iron levels from aliquots of samples analyzed by EPR are shown; at least six samplings from EPR samples after analysis were prepared and analyzed by ICP-MS

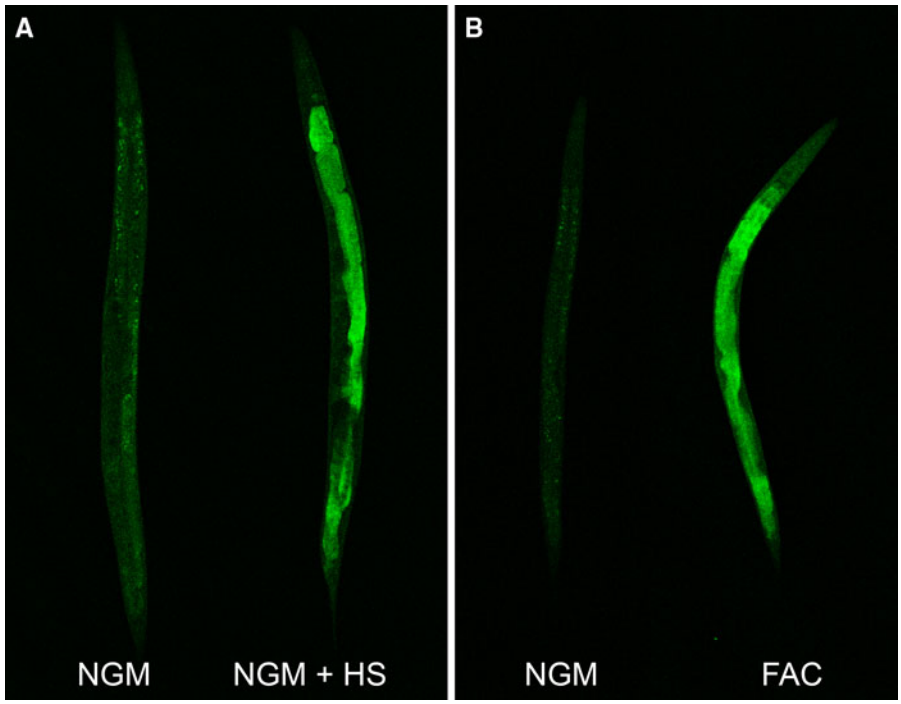

Fig. 6 Generalized oxidative stress is increased under conditions of elevated unincorporated and total iron. Representative images from non-iron supplemented control (NGM) and worms cultivated with 25 mM iron (25 FAC) provided for 2 days is shown in **B**. **A** shows a positive control, worms subjected to heat shock, 35 °C exposure for 4 h (NGM + HS), which is known to elevate ROS levels in vivo in comparison to non-heat stressed worms (NGM) grown at 22 °C. Worms were incubated in 10 μM 2′,7′-dichlorodihydrofluorescein diacetate for 30 min and then fixed on 2 % agarose pads containing 15 mM sodium azide on microscopic slides. Images were captured under the Leica TCS SP2 laser scanning confocal microscope set at an excitation wavelength of 488 nm and an emission wavelength of 510 nm. Data from three independent experiments (totaling 30 worms per condition) provided similar results; iron supplemented worms displayed significantly more DCF fluorescence in comparison to the control worms (*P* value as determined by *t* test for the change in fluorescence intensity with iron supplementation in comparison to untreated control was less than 0.03)

fluorescence was measured. Worms were cultivated mimicking the conditions used for Fe(III) EPR measurements. The worms grown on FAC medium displayed significantly higher DCF fluorescence (fold increase observed was 3.45 ± 1.16; Fig. 6b) indicating higher generalized ROS levels (Halliwell and Whiteman 2004) compared to control worms grown with no supplementation of iron. Positive control in panel A shows that with heat shock there is elevated levels of DCF fluorescence which is consistent with the well documented higher levels of oxidative stress known to be associated with heat shock.

Discussion

The low-temperature Fe(III) EPR at $g = 4.3$ methodology, which measures iron that can be captured by desferrioxamine, has provided a valuable tool for assessing the in vivo superoxide-induced stress status of yeast and *E. coli* (Srinivasan et al. 2000; Keyer and Imlay 1996). A positive correlation has been made between elevated "free" iron levels and superoxide stress in both model systems; *E. coli* and yeast lacking the superoxide scavenging enzymes SODs were shown to have higher "free" iron levels compared to wild-type. Additionally, in wild-type *E. coli* and yeast, treatment with the superoxide-generating redox-cycling drug, paraquat, resulted in a substantially enhanced iron EPR signal (Srinivasan et al. 2000; Keyer and Imlay 1996). In rats, elevated "free" iron measured by Fe(III) EPR at $g = 4.3$ directly correlated with elevated lipid peroxidation (Kozlov et al. 1996), while in yeast, a positive correlation between "free" iron and protein carbonyl levels was observed (Jensen et al. 2004) suggesting a strong

association between "free" iron and ROS-mediated macromolecular damage. Hence, we developed this methodology for *C. elegans* with the intention of exploring if the unincorporated iron captured by desferrioxamine measured by Fe(III) EPR at $g = 4.3$ could provide an indirect method for sensing ROS or ROS-mediated damage in vivo. We previously reported that *C. elegans* also produced an Fe(III) signal at $g = 4.3$, similar to *E. coli* and yeast (Pate et al. 2006). In that study, primary focus was on methodology development for the worms in addition to gaining some preliminary insights regarding the response of the Fe(III) EPR signal at $g = 4.3$ to ROS overproducing treatments.

Once the methodology was in place, our goals in this study were to uncover the factors that altered the unincorporated iron signal through the use of stressors that are known to elevate free radical levels. In particular, we wanted to determine if the unincorporated iron signal was simply responding to superoxide, as has been previously shown in yeast or to any ROS-overproducing treatment in general. Since our present work is modeled after studies carried out in *E. coli*, yeast and rats, and follows up on our previous study (Pate et al. 2006) with the goal of learning what factors modulate the $g = 4.3$ signal, we have focused our measurements on this g value only. It should be noted that aside from a resonance signal at $g = 4.3$ other resonances typically seen with $g = 4.3$ signals (as seen by (Hudder et al. 2007)) were not monitored in our study as we are interested in the $g = 4.3$ signal that has been tentatively assigned as adventitious iron(III) in yeast mitochondria by Hudder et al. The unincorporated iron signal responded to superoxide levels mediated by paraquat in wild-type *C. elegans,* as had previously been shown in other systems. However, it was heavily dependent on when the stress was placed on the system, the concentration or dose, and length of exposure. When exposed during early development the unincorporated iron signal was in fact lower. We believe that this reverse trend in the instance of early exposure could be due to an adaptive response mechanism that allows the worms to cope by upregulating protective factors. There is support for this theory in *C. elegans* (Darr and Fridovich 1995) and also in astrocytes, exposure to 0.2 mM paraquat for 24 h results in a 2-fold increase in catalase and a 3-fold increase in manganese SOD (MnSOD) mRNA levels (Rohrdanz et al. 2001). In an attempt to circumvent the induction of regulatory genes that could potentially mask real effects, we used developed worms and exposed them to stressors for shorter periods of time; we exposed L4 stage wild-type and short-lived *mev-1* worms to 1 mM or 100 mM paraquat for only 1 h. In both strains, under these conditions, a marginal but not statistically significant increase in the unincorporated iron signal was observed. While unexpected, there is some evidence that oxidative stress responses are observed only under certain concentrations. For instance, it has previously been shown that when oligochaete worms *Lumbriculus variegatus* were exposed to either 5 or 500 μg/μl of paraquat only marginal increases in protein oxidation levels were observed (Wiegand et al. 2007). Taken together, these results indicate that although the EPR signal responds to paraquat or superoxide radicals in particular, unincorporated iron levels increased only under certain conditions. Conditions that increased unincorporated iron also produced enhanced DCF fluorescence suggesting elevated ROS levels (data not shown). To be certain that the increases in unincorporated iron seen under these specific conditions were not simply due to an increase in overall iron levels in the worms, total iron levels were measured using ICP-MS. Data obtained indicate that the total iron levels were unchanged even when unincorporated iron levels increased suggesting that the observed increase was independent of new iron uptake into the cell.

We next wanted to test the EPR signal's response to other ROS generators, namely hydrogen peroxide. Although hydrogen peroxide does not contain any unpaired electrons and therefore it is believed to be less reactive than many other ROS, numerous effects of hydrogen peroxide exposure have been shown in various species. In wild-type yeast, a short-term (30 min) exposure to 2 mM hydrogen peroxide has been demonstrated to result in a 2-fold induction of the antioxidant proteins SOD and catalase compared to untreated controls (Baylaik et al. 2006). In L4 stage *C. elegans*, exposure to *Streptococcus pyogenes* and *Enterococcus faecium* that exclusively produce hydrogen peroxide, nematode death was observed within 24 h without the alteration in any antioxidant defense mechanisms. However, detoxification of hydrogen peroxide and death prevention has been demonstrated to occur only when exogenous catalase was added (Moy et al. 2004; Jansen et al. 2002). These results seem to suggest that L4 stage *C. elegans*, unlike yeast,

do not upregulate the hydrogen peroxide breakdown systems in response to this ROS. Our results showed that unincorporated iron levels increased significantly with a 30 min exposure to 2 mM hydrogen peroxide in wild-type *C. elegans* suggesting that the unincorporated iron measurements respond to hydrogen peroxide mediated oxidative stress as well. Overall, the results with paraquat and hydrogen peroxide treatment indicated that the unincorporated iron signal responds to superoxide and hydrogen peroxide. This is in line with Imlay and colleague's work (Jang and Imlay 2007), where the authors provide evidence that the 4Fe–4S cluster of aconitase that was thought to be sensitive only to superoxide until recently, is also sensitive to hydrogen peroxide.

Similar to chemical inducers of oxidative stress, including paraquat and hydrogen peroxide, thermal stress at 35 °C is also capable of evoking oxidative damage. However, unlike the previously used chemical pro-oxidants, temperature elevations result in the formation of not one specific kind, but all types of ROS in various cellular locations. Enhanced temperatures have been correlated with increased production of total ROS in isolated mitochondria (Heise et al. 2003). Thus, in order to determine if the unincorporated iron EPR signal would also respond to an increase in total ROS, we performed a series of heat shock experiments. In our earlier studies, we had shown that young *C. elegans* exposed to a short 1 or 2.5 h heat shock at 35 °C had normal or lower levels of unincorporated iron (Pate et al. 2006). *C. elegans* are capable of tolerating thermal stress through the induction of small heat shock proteins (hsps) and induction of the *C. elegans* hsp-16-2 has been shown to increase by 80 % within the first 2.5 h of exposure to 35 °C. The induction of these hsps are believed to be one of the reasons why *C. elegans* are able to adapt to changing temperature relatively quickly and do not die rapidly when conditions are altered (Link et al. 1999). Based on these findings, one may speculate that varied hsp induction response at varied time of exposure may be the reason why the initial studies showed no changes in unincorporated iron levels. Therefore, we opted to study the effects of a somewhat longer-term heat shock treatment of 4 h at 35 °C. We know that under our experimental conditions only after 6 h of exposure to 35 °C, a 40 % loss of viability is observed in wild-type worms. This allowed us to measure the unincorporated iron levels right before any heat induced death is typically observed. The results of this study showed that a significant increase in unincorporated iron pool is seen after 4 h of heat shock in both the wild-type and *mev-1* (short-lived superoxide overproducing) strains. In addition, we found a positive correlation between elevated unincorporated iron as measured by EPR after 4 h of heat shock with an increased ROS levels as observed using DCFDA (Fig. 6a). We also found that there was no change in total iron with long-term heat shock as compared to the control as determined by ICP-MS.

To continue understanding the response of unincorporated iron pool to various forms of ROS, we exposed worms from the L3 to L4 stage to various temperatures. Many studies have shown that alterations in temperature affect the growth of various organisms; in particular, *C. elegans* are highly sensitive to temperature and typically develop twice as fast at 25 °C as compared to 16 °C (Hope 1999; Byerly et al. 1976). Development is especially slowed when worms are exposed to lower temperatures beginning at the egg stage. This same slow developmental trend under lower temperature conditions has been observed in other species, along with a reduction in ROS, which is believed to be one of the reasons why some organisms have an increased life span at lower than ambient temperatures. For example, the wild-type *C. elegans* life expectancy has been shown to decrease from 42 to 12 days when the temperature is changed from 10 °C to 25 °C (Van Voorhies and Ward 1999). Therefore, we opted to test the effects of 12 h exposure to either higher or lower temperatures than normal. By first allowing all the worms to reach the L3 stage under the normal 22 °C, we minimized the drastic growth differences that would normally be observed if they were grown from eggs under specified lower or higher temperature. However, after 12 h of temperature exposure depending on the temperature used not all worms were in L4 stage. Worms cultured from L3 at 15 °C for 12 h did not reach L4, while 12 h at 25 °C produced young adults. In spite of this difference, results showed that when both wild-type and *mev-1* worms were grown at 15 °C, unincorporated iron levels were significantly lower. With a ten degree increase in temperature to 25 °C unincorporated iron signal increased (Fig. 2). These results are consistent with the theory of temperature mediated elevation in ROS. Additionally, these increased levels were also not a result of any change in total iron as determined

by ICP-MS. Taken together, the results show that the Fe(III) EPR unincorporated iron signal is responding to all radical mediated stress and does not simply elicit a response only to superoxide. In addition, a mutant worm strain *mev-1*, that has been demonstrated to produce increased ROS also had a two-fold increase in Fe(III) EPR signal in comparison to the wild-type. The increase seen was statistically significant (P value = 0.04; $n = 4$ samples prepared on 4 different days). Consistent with this finding DCF fluorescence of the *mev-1* strain was significantly higher than that of the wild-type worms (data not shown). These results are consistent with what is already known about the oxidative status of this strain. The total iron levels as measured by ICP-MS were nearly identical in both strains prepared side by side by growing them under identical conditions at this growth stage indicating that the increase in unincorporated iron levels were not due to an increase in total iron in this strain.

In nearly all of our nematode investigations, we have used L4 staged young *C. elegans* since large numbers of worms (over 10,000) are required for EPR measurements. As it is well characterized that elevated damage to biomolecules mainly occurs during the last 1/3rd of an organisms life-span (Levine and Stadtman 2001), we investigated the effect of age on levels of unincorporated iron. Since unincorporated iron levels correlate with lipid peroxidation and protein carbonyl levels in other organisms, we compared 5 day old adults with L4 stage worms. There was a statistically significant increase in unincorporated iron with age with no significant alteration in the total iron levels. Due to the high numbers of worms needed for the analysis and the fact that decreasing percentages of worms are alive late in life, we chose to investigate 5 day-old worms. These data certainly suggest that with age, where there is significant evidence demonstrating the build-up of ROS-mediated damage, there is an increase in the unincorporated iron pool.

To understand the contribution of iron availability to the unincorporated iron signal, worms were grown on plates containing 25 mM FAC. We used 25 mM FAC based on the published work (Kim et al. 2004), and surprisingly this concentration decreased the life-span of the wild-type worms only slightly. However, under these conditions we saw a significant increase in total iron accompanied by a greatest fold increase in unincorporated iron observed for *C. elegans*. Iron being a redox-active metal is capable of participating in Fenton type reactions which can lead to more ROS production in vivo. In fact, we noticed higher levels of generalized oxidative stress in vivo under these conditions as measured using DCF fluorescence showing a positive correlation between total iron, unincorporated iron and ROS levels.

Conclusion

The results presented here demonstrate that the Fe(III) EPR at $g = 4.3$ based unincorporated iron levels can provide a marker of oxidative stress status in vivo provided there is no change in total cellular iron levels between the samples compared. However, induced oxidative stress may also lead to iron mobilization which can be seen as EPR signal at $g = 4.3$. Hence, unincorporated iron observed can be a consequence of oxidative stress but also serve as a marker of oxidative stress. On the other hand, if total iron levels change, an increase in internal total iron can contribute to increased labile iron pool and thus may produce higher concentration of unincorporated iron. Alternatively, increased cellular iron could lead to elevated ROS production which could result in higher levels of unincorporated iron. Nonetheless, it is interesting to note that this pool of unincorporated iron is modulated by both iron availability and generalized ROS status. Studies in other systems have referred to this type of iron pool as low molecular weight, labile, transient, adventitious, "free" and/or chelatable pool of iron; currently it remains unclear if these forms of iron are all one and the same. Although increased unincorporated iron levels were observed with various stressors, the cellular location and the chemical identity of this $g = 4.3$ EPR-detectable iron is still vague. Studies in *E. coli* have shed some light on the chemical nature of the low molecular weight mobile iron pool and it was shown to contain a novel ferrous iron binding carbohydrate phosphate (Bohnke and Matzanke 1995). Since yeast mitochondria also contain this EPR-detectable iron at $g = 4.3$ it is possible this is the iron in transit; the details of these questions remain largely unanswered in more complex multicellular systems including the nematodes.

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