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Disruption of Iron Homeostasis Increases Phosphine Toxicity in *Caenorhabditis elegans*

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The aim of this study is to identify the biochemical mechanism of phosphine toxicity and resistance, using *Caenorhabditis elegans* as a model organism. To date, the precise mode of phosphine action is unclear. In this report, we demonstrate the following dose-dependent actions of phosphine, *in vitro*: (1) reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), (2) release of iron from horse ferritin, (3) and the peroxidation of lipid as a result of iron release from ferritin. Using *in situ* hybridization, we show that the ferritin genes of *C. elegans*, both ferritin-1 and ferritin-2, are expressed along the digestive tract with greatest expression at the proximal and distal ends. Basal expression of the ferritin-2 gene, as determined by quantitative PCR, is approximately 80 times that of ferritin-1. However, transcript levels of ferritin-1 are induced at least 20-fold in response to phosphine, whereas there is no change in the level of ferritin-2. This resembles the reported pattern of ferritin gene regulation by iron, suggesting that phosphine toxicity may be related to an increase in the level of free iron. Indeed, iron overload increases phosphine toxicity in *C. elegans* at least threefold. Moreover, we demonstrate that suppression of ferritin-2 gene expression by RNAi, significantly increases sensitivity to phosphine. This study identifies similarities between phosphine toxicity and iron overload and demonstrates that phosphine can trigger iron release from storage proteins, increasing lipid peroxidation, leading to cell injury and/or cell death.

Key Words: **Phosphine; ferritin; iron overload; longevity; oxidative stress; *C. elegans*.**

Phosphine, phosphorous hydride (PH_3), has been used worldwide for protecting stored products from insect infestation since the 1930s, with extensive use for the last several decades (Chaudhry *et al.*, 2004). Phosphine has a range of physical and chemical properties that make it useful as a fumigant, the most important of which are that it is highly toxic to actively respiring aerobic organisms and is a gas under standard conditions. Due to a density similar to air, phosphine can diffuse rapidly throughout a grain bulk without active circulation (Chaudhry, 1997). Studying the mechanism of phosphine toxicity has recently received significant attention due to two factors: the emergence of high-level resistance toward phosphine among several pest insects of stored products and the recent global ban on use of the alternative fumigant methyl bromide, which causes ozone depletion (Fields and White, 2002).

It was proposed initially that phosphine could inhibit mitochondrial respiration by interaction with cytochrome *c* oxidase sufficient to cause lethal depletion of ATP (Nakakita, 1976). However, the magnitude of respiratory inhibition that was observed *in vitro* could not be replicated *in vivo* (Price, 1980). It was subsequently proposed that partial inhibition of the mitochondrial electron transport chain could enhance generation of superoxide which could rapidly dismutate to hydrogen peroxide (Bolter and Chefurka, 1990). The possible role of hydrogen peroxide in phosphine toxicity was elaborated in a recent chemical model in which phosphine and hydrogen peroxide were proposed to react to generate hydroxyl radical. The hydroxyl radical was proposed to initiate lipid peroxidation resulting in cell damage and possible death (Quistad *et al.*, 2000).

Any mechanism of phosphine toxicity must address two themes that are consistent in phosphine toxicity: not only is oxygen essential (Kashi, 1981) and synergistic (Cheng *et al.*, 2003) but also lipid peroxidation is a consequence of phosphine exposure both *in vitro* (Hsu *et al.*, 1998; Quistad *et al.*, 2000) and *in vivo* (Hsu *et al.*, 2000, 2002b; Quistad *et al.*, 2000). Superoxide and hydrogen peroxide can both induce lipid peroxidation, but they must first be converted by transition metals such as iron to hydroxyl radical *via* the Fenton (Equation 1) and Haber-Weiss reactions (Equation 2):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\circ} + OH^{-}. \quad (1)$$

$$O_2^{-} + H_2O_2 + Fe^{2+} \rightarrow O_2 + OH^{\circ} + OH^{-}. \quad (2)$$

Alternatively, oxoiron species, such as the perferryl (Fe^{2+}-O_2) and ferryl (Fe^{2+}-O) ions, can serve as potent initiators of lipid

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peroxidation (Qian and Buettner, 1999). Regardless of the source of reactive oxygen, iron is thought to be a major contributor to lipid peroxidation.

Due to its toxicity, levels of free iron are tightly controlled, with the level of free iron in an organism are normally maintained at an extremely low level, about 10^{-8}M and 10^{-18}M for Fe^{2+} and Fe^{3+}, respectively (Harrison and Arosio, 1996). Iron sequestration is primarily the responsibility of ferritin, a hollow protein of 24 subunits, that concentrates iron (Fe^{3+}) as an insoluble ferrihydrite. The structure of ferrihydrite varies within the iron core of the ferritin molecule, ranging from highly crystalline to amorphous, depending on the amount of included phosphate as well as the rate of formation (Harrison and Arosio, 1996). Because iron is an essential component of many redox-sensitive enzymes and carrier molecules, an iron storage molecule such as ferritin must also be able to release the iron as it is required.

Mobilization of iron from ferritin, *in vivo*, is unclear, but *in vitro* experiments demonstrate that iron can be released by reductants. Phosphine is a potentially significant reducing compound with a redox potential of − 1.18 V relative to Fe^{3+} in ferritin of − 0.19 V. The possibility that perturbation of iron homeostasis could play a role in phosphine toxicity has not been suggested previously, but iron released from ferritin is known to induce oxidative damage (Reif, 1992). We observed that transcripts from the gene encoding the ferritin heavy-chain protein increased approximately 24-fold in response to phosphine (unpublished microarray experiment). An identical observation had previously been shown for iron (Gourley *et al.*, 2003). We therefore proposed that release of iron from ferritin by phosphine could increase lipid peroxidation, leading to cell damage. We have now tested this hypothesis both *in vitro* and *in vivo*, using *Caenorhabditis elegans* as a model organism.

MATERIALS AND METHODS

Escherichia coli and nematode strains. The wild-type *C. elegans* strain, N2 (var Bristol), and its food, the *Escherichia coli* strain OP50, were kindly provided by Dr Warwick Grant, La Trobe University, Australia. Unless specified, all nematodes were cultured at 20°C on NGM (18 g/l agar, 2.5 g/l peptone, 3 g/l NaCl, 5 mg/l cholesterol, 1mM $CaCl_2$, 1mM $MgSO_4$, and 25mM KH_2PO_4, pH 6) seeded with *E. coli* strain OP50.

Phosphine generation. Phosphine (Bayer Australia Ltd, Australia) was generated by dissolving an aluminum phosphide tablet in a solution of 5% sulfuric acid. The concentration of phosphine was determined by gas chromatography, using nitrogen (N_2) as a standard and Freon-24 as a carrier. Unless indicated, phosphine exposures were performed in desiccators fitted with a septum through which phosphine could be injected to the desired concentration.

Ferric chloride reduction by phosphine. The reactions were conducted in 5-ml sealed screw cap vials. Each vial contained 1 ml of buffer (230mM sodium acetate pH 5.6, 0.5mM ferrozine [Sigma, St Louis, MO], and 0.1mM of ferric chloride [Sigma]). These vials were injected with 0, 10, 20, 50, and 100 μl of phosphine, respectively. Reactions were left at 23°C for 8 h before detecting ferrous-ferrozine complexes at 562 nm, using a visible spectrophotometer (Hitachi U-1100). A previous time course experiment had indicated that 8-h exposure was in a linear range of phosphine-reduced ferric chloride. Ferrous ammonium sulphate (Sigma) was used as a standard.

Phosphine-mediated release of iron from ferritin. The reactions were conducted in 24-well plates. Each well contained 75mM sodium acetate pH 5.6, 1mM ferrozine, and 0.2 mg ferritin (Sigma) in a 1-ml reaction. All 24-well plates were placed in desiccators. The control desiccators were injected with air, and the treatment desiccators were injected with phosphine to a concentration of 1, 2, and 4 mg/l, respectively. The volume of the chambers was measured to allow final phosphine concentrations to be calculated precisely. The final phosphine concentration in the desiccators was not measured directly. Reactions were left at 25°C for 12 h before reading the absorbance at 562 nm, using ferrous ammonium sulphate as a standard. A previous time course experiment had indicated that a 12-h exposure was in a linear range of iron release from ferritin by phosphine.

Determination of lipid peroxidation by the thiobarbituric acid reaction. The reactions were conducted in 24-well plates. Each well contained a 1-ml mixture of 10 μl of cod liver oil (Sigma), 890 μl of 0.2% Triton X100 in 100mM sodium acetate buffer, pH 5.6, and 100 μl of 2 mg/ml ferritin. All 24-well plates were then placed in desiccators. The control desiccators were injected with air, and the treatment desiccators were injected with phosphine to a concentration of 4 mg/l. Reactions were left at 25°C for 24 h. The 24-h exposure time was selected as it was in the linear range of iron release from ferritin and was also consistent with the design of a subsequent *in vivo* experiment. A 50-μl volume of each reaction then was taken for quantification of lipid peroxidation products using the thiobarbituric acid (TBA, Sigma) method (Mihara and Uchiyama, 1978). Briefly, the total 1.5 ml solution (50 μl of sample, 0.5 ml of 0.6% TBA, 0.1 ml of 8.1% SDS, 0.1 ml of deionized water (Milli Q), and 0.75 ml of 1% phosphoric acid) was heated in a heat block containing glycerine at 95°C for 45 min. Then samples were cooled rapidly in an ice bath for few min. After that, TBA reactants were extracted by adding 800 μl of TBA reaction into 800 μl of *n*-butanol. The samples were mixed by shaking and then separated by centrifugation at 4000 × g (Pathtech, Spectrafuge16M, Pathtech, Australia). Absorbance of the red colored aqueous layer, due to TBA adduct compounds, was measured by spectrophotometer (Softmax Prov. 2.2.1, Molecular Devices Corp, Sunnyvale, CA) at 532 nm. 1,1,3,3-Tetramethoxypropane was used as a standard.

Tissue preparation for in situ hybridization. At 42 h of age, nematodes (N2) were divided into treatment and control groups. The controls were exposed to air, whereas the treatment nematodes were exposed to 0.3 mg/l phosphine for 5 h at 23°C. A 5-h exposure was selected to be an early stage of phosphine exposure prior to secondary effects. This time point was also consistent with the original microarray experiments in which induction of the ferritin-1 gene was first observed. The nematodes then were washed off the plates with M9 buffer prior to sucrose floatation which separated the living nematodes from bacteria and other debris. Then they were washed three times in 0.1M NaCl and three times with washing buffer (100mM HEPES-KOH, pH 7.5, 1mM $MgSO_4$, and 0.2mM EGTA). After that, nematodes were fixed in 4% formaldehyde in washing buffer for 4 h at room temperature. Following fixation, they were dehydrated in a methanol series from 20 to 100% in 20% increments. Samples were subjected to 10 min of gentle rotation per step prior to centrifugation and removal of the liquid. After dehydration, the nematodes were stored in 100% ethanol at − 80°C, until they were used.

Synthesis of single-stranded DNA probes for in situ hybridization. Nematodes were washed off the plates with 0.1M NaCl and separated from bacteria and other debris by sucrose floatation. Samples were washed three times with cold 0.1M NaCl before being transferred to fresh 1.5 ml tubes. After that, 1 ml Trizol reagent (Invitrogen, Carlsbad, CA) was added to each 0.2 ml nematode pellet. Samples were mixed, flash frozen in liquid N_2, and kept at − 80°C until RNA extraction. To extract total RNA, samples were warmed to room temperature (23°C). The TRIZOL reagent was removed by chloroform extraction and the RNA precipitated with isopropanol as recommended by the

manufacturer. First-strand cDNA was generated as recommended by Invitrogen using random primers and superScript II Reverse Transcriptase. The target cDNA sequences then were amplified by PCR, using specific forward and reverse primers–5′ AAGCTTCATGGAATCGCCG 3′ and 5′ AATATTCTCC-GAGTCCTGGGC 3′ for the *ftn-1* gene and 5′ CGCTTCTGAAGTTGCACTCG 3′ and 5′ CCAACTCCTGGTC CGACTCTC 3′ for the *ftn-2* gene. The target PCR products were purified by gel electrophoresis, using the Nucleospin gel purification kit. Probe templates were confirmed initially by size and later by DNA sequencing. The PCR DIG probe synthesis kit (Roche Applied Science, Germany) then was used for synthesis of nonradioactive, single-stranded probes.

RNA in situ hybridization and detection. The spatial distribution of ferritin mRNA was determined using *in situ* hybridization as previously described (Seydoux and Fire, 1995). Hybridization was carried out at 37°C overnight. DIG-labeled probe was detected using an anti-DIG antibody conjugated to alkaline phosphatase according to the manufacturer's instructions (Boehringer Mannheim, Germany). The enzymatic reaction of alkaline phosphatase and its substrates was carried out, using 1-Step NBT/BCIP solution (Pierce, Rockford, IL). Hybridization was visualized using a Nikon Microphot-FXA microscope.

Dose effect of phosphine exposure on ferritin expression. Nematodes were grown for 42 h on NGM plates seeded with *E. coli* strain OP50. Then they were exposed to phosphine at a range of concentrations (0, 0.15, 0.3, 0.45, and 0.6 mg/l) for 10 h at 20°C. A preliminary time course experiment was carried out to determine the time of maximal *ftn-1* expression which was identified as 10 h. Following phosphine exposure, total RNA was extracted as described above for probe synthesis for *in situ* hybridization. Ferritin mRNA levels were determined by quantitative real-time PCR, using *rpl-15*, a ribosomal protein transcript, as an internal control.

Construction of plasmids for RNAi of ferritin genes. The ferritin-1 gene sequence was amplified by the PCR technique using the forward primer, 5′ TGTCGAACAAATATTCTCCGAGT 3′, and reverse primer, 5′ TTTTCT GCCAAGTAAATCGTCAT 3′. PCR products were separated by gel electrophoresis and purified by gel purification kit (Qiagen, Valencia, CA). The 1020-bp PCR fragments were then cloned into the pGEM-T vector (Promega, Madison, WI) which was transformed into *E. coli*, strain DH5α *via* electroporation. The integrity of selected clones was confirmed by PCR and digestion with *Spe*I (Biolabs, Ipswich, MA) and *Sac*II (Biolabs). The fragments were then excised from pGEM-T with *Spe*I and *Sac*II and ligated into pLT440, the cloning site of which is flanked by promoters for T7 polymerase which drive high-level synthesis of double-stranded RNA. The host strain of *E. coli*, HT115, contains a gene encoding T7 RNA polymerase under the control of the *Lac* operon promoter which drives the production of high levels of double-stranded RNA. HT115 also contains an RNase III deficiency that prevents degradation of the dsRNA once it is formed (Kamath *et al.*, 2000). The integrity of this clone was confirmed by DNA sequencing, and the strains were stored at − 80°C in growth medium containing 15% glycerol. The ferritin-2 RNAi plasmids were constructed as described for ferritin-1, except that different primers were used. The 1006-bp fragment of *ftn-2* was amplified using 5′ TTAAAGACCAAAC GGCTTATCAA 3′ (forward) and 5′ CTTGATTTTTGCCATTTTCTGAC 3′ (reverse) primers.

Ferritin suppression and phosphine toxicity in C. elegans. *Escherichia coli* strains containing the RNAi vectors were cultured in 100 ml LB medium at 37°C with shaking. After overnight incubation (14–16 h), bacteria were harvested by centrifugation at 5000 × g (Beckman, Model J2-21) at 4°C for 15 min. Then bacteria were seeded on NGM plates containing 1mM IPTG and 100 μg of ampicillin and left at 23°C overnight to allow generation of double-stranded RNA (Kamath *et al.*, 2000). For each treatment, the nematodes were cultured on NGM plates seeded with bacteria in one of the following combinations: (1) bacteria transformed with vector alone that did not produce dsRNA, (2) bacteria that produced dsRNA from the *ftn-1* gene, (3) bacteria that produced dsRNA from the *ftn-2* gene, or (4) a mixture of the last two *E. coli* strains. Wild-type nematodes at larval stage 2 were transferred to these plates and were allowed to grow for 42 h before they were transferred to 12-well plates seeded with the same type of *E. coli* on NGM medium supplemented as before. Plates were divided into two groups, control and treatment. The control groups were exposed to air, and the treatment groups were exposed to phosphine at a concentration of 0.1, 0.15, or 0.2 mg/l for 24 h at 25°C. The mortality rates of the control and treatment groups were investigated after phosphine exposure. For nematodes cultured at 20°C, our standard protocol is to fumigate nematodes at 42 h of age for a 24-h treatment period. Given the rapid development cycle of the nematodes, this provides adequate time to assess the mortality phenotype prior to the offspring of the treated worms interfering with the count.

Confirmation of gene suppression by quantitative real-time PCR. Quantitative real-time PCR was performed using an ABIPrism 7000, sequence detector system with SYBR Green (Applied Biosystems, Foster city, CA) as the fluorescent reporter. A 250-μl PCR reaction contained 5 μl of specific primers (1μM for *ftn-1* and control gene; 2μM for *ftn-2*), 2.5 μl water (Pharmacia & Upjohn, Kalamazoo, MI), 12.5 μl of 2× SYBR Green PCR master mixes and 5 μl of cDNA (10 ng). The primer sequences for ferritin-1 are 5′ GCGGCCGTCAA-TAAACAGATT 3′ (forward) and 5′ GATATCATCACGATCGAAGTGTGC 3′ (reverse). The primer sequences for ferritin-2 are 5′ TGCCGTTAACAAGCAG ATCAA 3′ (forward) and 5′ GAAGGGCAACATCGTCACG 3′ (reverse). The primer sequences for control gene (*rpl-15*) are 5′ TACCGTGCCAAGCAA GGATT 3′ (forward) and 5′ TGGCTTTCCGTAGGTTTGTCC 3′ (reverse). The DNA amplification used the following conditions: an initial denaturation step at 95°C for 10 min and 45 cycles of amplification at 95°C for 15 s and 60°C for 60 s.

Iron overload and phosphine toxicity in C. elegans. Freshly prepared 0.5M ferrous ammonium sulphate was added to NGM agar to a concentration of 0, 1.5, 3, or 4.5mM iron. NGM plates were prepared in 12-well micro titer trays which were seeded with *E. coli* strain OP50 one day before nematode transfer. Wild-type nematodes (N2) aged 24 h were transferred to two 12-well trays containing NGM agar of each iron concentration. The nematodes were allowed to grow on these media for a period of 24 h prior to exposure of one tray to air and the other to air containing 0.3 mg/l phosphine at 20°C for 24 h. Mortality rates on control plates (exposed to air only) were investigated immediately after the 24-h treatment period, whereas mortality due to phosphine exposure had to be delayed by 24 h. The delay was necessary because phosphine has an anesthetic effect that results in immobilization of individuals that later show complete recovery from phosphine exposure. It was not possible to delay assessment of the control individuals due to the confounding effects of offspring production on mortality assessment in the air-treated controls. We observed that production of offspring was not a problem with the phosphine-treated nematodes because phosphine exposure causes developmental arrest that delays offspring production. Our experimental design reflects a compromise between ensuring an adequate period of exposure to phosphine while allowing the bulk of development to occur in the absence of excess iron followed by 24 h of iron exposure according to Gourley (Gourley *et al.*, 2003). We also had to allow time for the standard 24-h exposure to phosphine prior to the offspring growing up and interfering with counting.

Statistical analysis. The effect of phosphine exposure on RNAi-treated animals was analyzed by two-way ANOVA, whereas the effect of iron on phosphine toxicity was analyzed by one-way ANOVA. The effect of phosphine on lipid peroxidation was analyzed by Student's *t*-test. The threshold for statistic significance was set at $p < .05$.

RESULTS

Phosphine Can Reduce Ferric Iron

In the presence of reducing agents, ferric iron can be reduced to the ferrous form that can be detected as a chromophore complex with ferrozine, an iron chelator. Ferrozine has a high affinity toward the ferrous ion and forms a magenta colored

complex in a 3:1 ratio between ferrozine and ferrous iron ion (Stookey, 1970). The quantity of the ferrozine-ferrous iron complex was determined by measuring absorbance of the reaction mixture at a wavelength of 562 nm. The concentration of ferrous iron was calculated, using ferrous ammonium sulphate as a standard. As shown in Table 1, phosphine is able to reduce ferric ion to ferrous ion in a dose-dependent manner at a rate of 1 μmol Fe^{3+} reduced per 52 μmol PH_3 over the course of the 8-h exposure. Given the poor solubility of phosphine, its concentration in the airspace of the vials is estimated to be about 4.5 times greater than in the aqueous solutions.

Phosphine-Mediated Release of Iron from Ferritin Is Concentration Dependent

The reduction of iron (Fe^{3+} to Fe^{2+}) is a prerequisite for the release of stored iron from ferritin (Loehr, 1989). As such, the capacity of phosphine to reduce ferric iron indicates that phosphine also may be able to promote the release of iron from ferritin. The results presented in Table 2 demonstrate that phosphine is indeed able to release iron from ferritin in the presence of a colorimetric iron chelator. Release of iron from ferritin occurs in a dose-dependent manner, with release of approximately 0.25 nmol iron per hour from 0.2 mg ferritin in the presence of 4 mg/l phosphine (0.25μM iron per hour in a 1-ml reaction). This level of release is approximately twice the rate of leakage in the control reactions.

Phosphine Increases Lipid Peroxidation in the Presence of Ferritin

As shown in Table 3, a significant level of lipid peroxidation, measured indirectly as 2μM thiobarbituric acid reactive substances (TBARS), occurred when the ferritin-lipid mixture was exposed to air for 24 h. When the ferritin-lipid mixture was exposed to phosphine for 24 h, a significant increase in TBARS (2.7μM) relative to the air-only control was observed ($p < .003$, *t*-test). This is consistent with a phosphine-induced increase in free iron relative to the air-only treatment, with the released iron available to catalyze lipid peroxidation.

TABLE 1
Concentration-Dependent Reduction of Ferric Chloride by Phosphine

Phosphine concentration (mg/l)	Iron reduced (μM) (means ± SEM)
0	0
0.51	0.31 ± 0.06
1.05	0.67 ± 0.03
2.62	1.48 ± 0.04
5.27	2.54 ± 0.17

Note. Data are means ± SEM.

TABLE 2
Concentration-Dependent Release of Iron from Ferritin by Phosphine

Phosphine concentration (mg/l)	Iron released (μM) (means ± SEM)
0	1.37 ± 0.19
1	2.13 ± 0.22
2	2.85 ± 0.14
4	3.32 ± 0.24

Note. Data are means ± SEM.

Spatial Distribution of Ferritin Gene Expression

Under the conditions employed, basal expression of the ferritin-2 gene showed a broad spatial distribution of gene expression, predominantly, in the intestinal tract of adult nematodes, with the highest expression observed at the proximal and the distal ends (Fig. 1c). Unlike ferritin-2, the basal expression of the ferritin-1 gene was so low that it was difficult to determine the basal distribution of gene expression (Fig. 1a). Phosphine treatment of the nematodes revealed a dramatic increase in ferritin-1 transcript expression (Fig. 1b), whereas a similar signal intensity was not observed in the air-treated control nematodes (Fig. 1a). The distribution of ferritin-1 gene expression following induction by phosphine closely resembled the distribution of ferritin-2 gene expression. Interestingly, ferritin-2 transcript expression did not appear to be significantly induced by phosphine exposure and the distribution of gene expression remained unchanged as well (compare Figs. 1c and 1d).

Dose Dependence of Ferritin Gene Induction by Phosphine

Quantitative real-time PCR indicated a clear dose-dependent increase in ferritin-1 transcript in response to phosphine (Fig. 2). The linear regression analysis confirmed that ferritin-1 expression is correlated with phosphine concentration ($R^2 = 0.582$, $p < .0005$). In contrast to ferritin-1, ferritin-2 transcript was not induced by phosphine regardless of the dose of phosphine applied (0.15–0.6 mg/l) over a period of 10 h. However, the

TABLE 3
Lipid Peroxidation Due to Phosphine-Mediated Release of Iron from Ferritin

Condition	TBARS (μM) (means ± SEM)
Air exposure	2.08 ± 0.09
Phosphine exposure (0.3 mg/l)	2.69 ± 0.12*

Note. Data are means ± SEM.
*$p < .003$ for air exposure versus phosphine exposure.

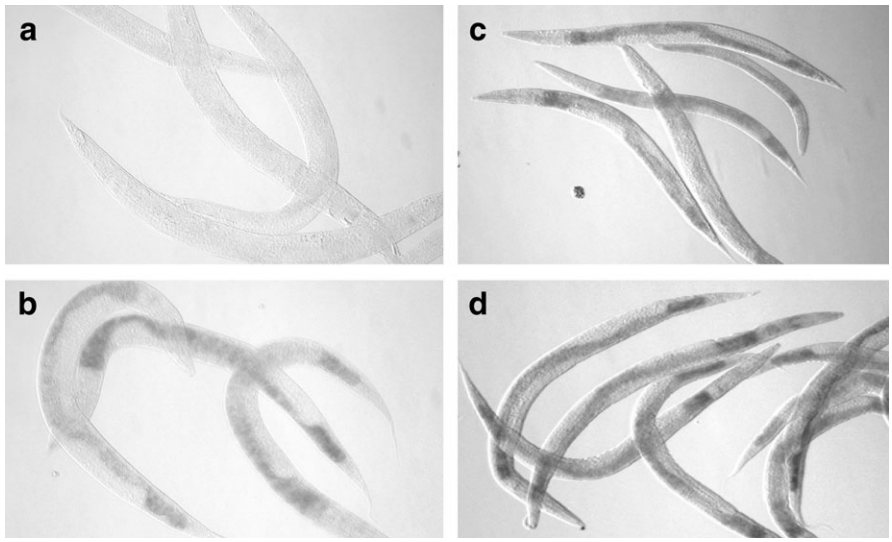

FIG. 1. Effect of phosphine on expression of ferritin genes in adult nematodes. (a) Expression of the ferritin-1 gene in control nematodes exposed to air. Width of figure = 0.8 mm. (b) Expression of the ferritin-1 gene after a 5-h exposure to phosphine at 0.3 mg/l. Width of figure = 1.0 mm. (c) Expression of the ferritin-2 gene in control nematodes exposed to air. Width of figure = 1.2 mm. (d) Expression of the ferritin-2 gene after a 5-h exposure to phosphine at 0.3 mg/. Width of figure = 1.1 mm.

basal level of ferritin-2 transcript is 87 times higher than the basal level of ferritin-1 as well as several times higher than that of ferritin-1 following induction by phosphine.

Ferritin-2 Suppression Causes Increased Phosphine Susceptibility

RNAi was used to suppress the ferritin-1 and ferritin-2 genes, either individually or together, for a period of 3 days. The magnitude of gene suppression was determined by quantitative real-time PCR, as shown in Figure 3. Comparison of transcript abundance for the two genes in the control nematodes indicated that the basal level of ferritin-1 gene expression is approximately 150 times lower than that of ferritin-2. Surprisingly, the ferritin-1 gene suppression experiment did not cause any significant change in transcript levels of either ferritin-1 or ferritin-2 relative to the control. In contrast, ferritin-2 gene suppression caused a dramatic reduction in ferritin-2 transcript to 10% of that in the control (Fig. 3). Similarly, an experiment designed to suppress both genes simultaneously resulted in no suppression of ferritin-1 but significant suppression of ferritin-2. The suppression of ferritin-2 transcript levels was not as efficient, though it was reduced to less than 20% of that observed in the control.

Despite dramatic effects on the abundance of transcript from the ferritin-2 gene, there was no observable effect on the development, behavior, or health of the treated nematodes. The effects of ferritin gene suppression on phosphine toxicity in *C. elegans* was then assessed (Fig. 4). As expected, two-way ANOVA analysis indicated that phosphine has a highly significant effect on nematode mortality ($p < .0005$), regardless of ferritin transcript abundance. As also might be expected for such an important gene, reduction in abundance of the ferritin-2 transcript caused a significant increase in mortality, regardless of phosphine application ($p = .003$). Interestingly, there also was a barely significant interaction between phosphine toxicity and ferritin-2 suppression ($p = .05$). Consistent with the failure of ferritin-1 gene suppression, no effect of the ferritin-1 gene on mortality could be observed. Neither was there any significant interaction between phosphine and ferritin-1 gene suppression nor even between phosphine treatment and simultaneous ferritin-1 and ferritin-2 gene suppression.

Iron Overload Increased Phosphine Toxicity

Ferritin is known to act as a buffer that prevents the cellular availability of toxic levels of free iron. Given the previous

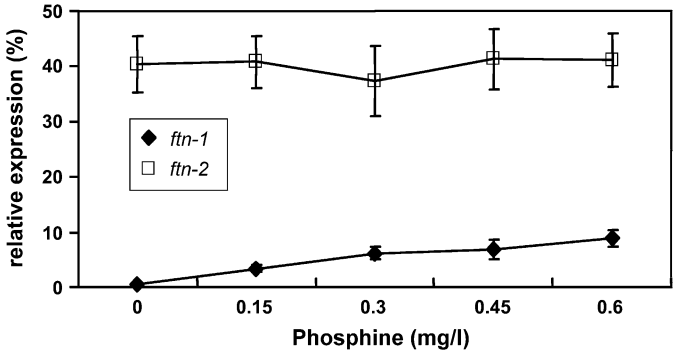

FIG. 2. Induction of the ferritin genes by phosphine. Adult nematodes (42 h after hatching) were exposed to phosphine at varying concentrations (0, 0.15, 0.3, 0.45, or 0.6 mg/l) for 10 h at 20°C. The level of ferritin mRNA as determined by quantitative real-time PCR is expressed as the mean ± SEM of five replicates with *ftn-1* (♦) and *ftn*-2 (□).

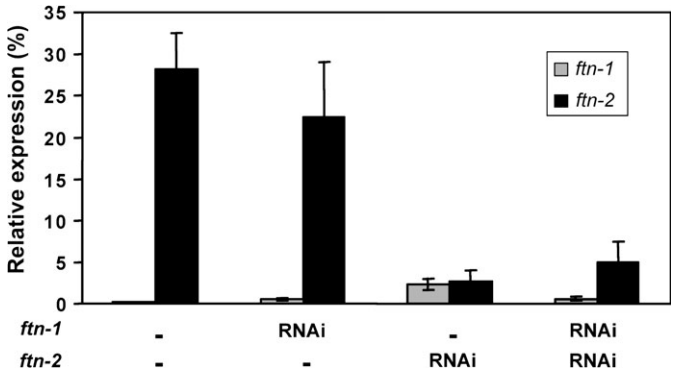

FIG. 3. Suppression of ferritin mRNA levels by RNAi. Nematodes at larva stage 2 were grown for 3 days on NGM plates seeded with *E. coli,* HT115, containing an unmodified vector or vectors designed to suppress either ferritin-1 or ferritin-2. In one of the treatments, nematodes were fed a combination of *E. coli* designed to suppress ferritin-1 and *E. coli* designed to suppress ferritin-2. The level of ferritin mRNA, as determined by quantitative real-time PCR, is presented as the mean ± SEM of three replicates ($n = 3$).

observation that suppression of the ferritin-2 gene resulted in heightened sensitivity to phosphine, we wished to determine whether the complementary experiment, overloading the buffering capacity of the available ferritin, would similarly result in heightened sensitivity to phosphine. As shown in Figure 5, there is indeed a correlation between iron overload and phosphine toxicity in *C. elegans*, with higher iron concentration associated with a higher mortality rate following phosphine exposure ($p = .003$, one-way ANOVA). Iron overload in the absence of phosphine exposure did not result in a statistically significant increase in mortality at any of the concentrations used in this study.

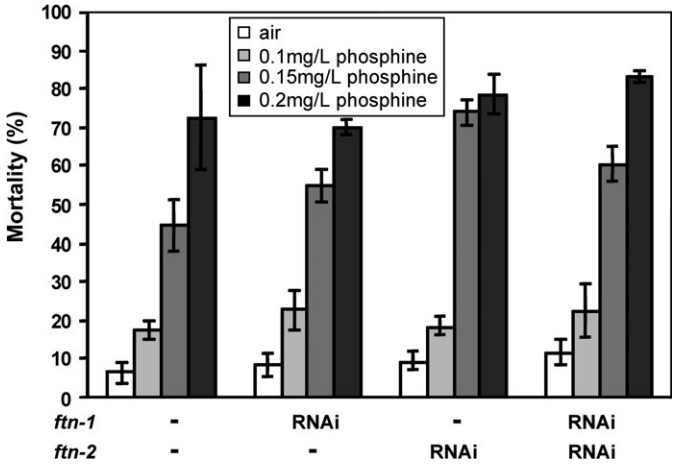

FIG. 4. Effect of ferritin suppression on phosphine toxicity in *Caenorhabditis elegans*. Nematodes at larval stage 2 were grown on NGM agar plates seeded with *Escherichia coli* strain HT115 with or without ferritin gene suppression vectors for 42 h. They then were exposed to air or air containing phosphine at the indicated concentrations. Results are presented as the mean ± SEM of four replicates ($n = 4$).

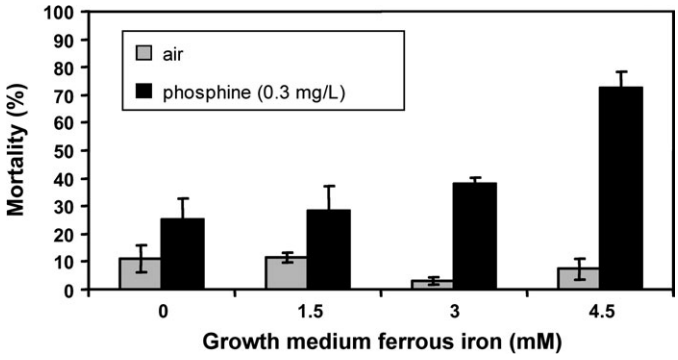

FIG. 5. Iron overload increases phosphine toxicity in *Caenorhabditis elegans*. At 24 h after hatching, nematodes were transferred to NGM containing 0, 1.5, 3.0, or 4.5mM ferrous iron. Nematodes were left to grow for 24 h prior to exposure for 24 h to 0 or 0.3 mg/l phosphine in air at 20°C. The mortality results are presented as the mean ± SEM of three replicates ($n = 3$).

DISCUSSION

Several lines of evidence point to oxidative stress playing a significant role in the toxicity of phosphine. It is clear that phosphine requires oxygen for its toxicity (Bond *et al.*, 1969), which is consistent with the observed oxidation of lipids following phosphine exposure (Hsu *et al.*, 1998, 2000). The ability of phosphine to cause oxidative stress is paradoxical, however, as phosphine is a reducing agent (Chaudhry, 1997). As a result, phosphine has been proposed to cause oxidative stress indirectly by perturbing electron flow through the mitochondrial respiratory chain, which results in excessive generation of the reactive oxygen molecule, superoxide (Bolter and Chefurka, 1990). Superoxide alone, however, is not sufficient to initiate oxidation of lipid (Cheeseman, 1993) and neither can hydrogen peroxide generate lipid radicals unless transition metal ions such as iron are present (Minotti and Aust, 1987).

As a transition metal, iron is highly reactive and therefore essential to the catalytic activity of a large number of enzymes. The reactivity of iron means that its availability must be tightly regulated. Special iron storage proteins, such as ferritin, ensure that the concentration of free iron remains low but that iron is available when required. Some reducing compounds have been found to induce the release of iron from iron storage proteins *in vitro* (Sirivech *et al.*, 1974). As phosphine is a reducing compound, we surmised that release of iron from ferritin by phosphine may contribute to lipid peroxidation following phosphine exposure. The present study demonstrates that phosphine is able to reduce ferric iron to the ferrous form and is indeed able to release iron from ferritin as we proposed.

Many chemicals such as paraquat (Thomas and Aust, 1986), humic acid (Ho *et al.*, 2003), and arsenic species (Ahmad *et al.*, 2000) have been demonstrated to be toxic, partially due to their ability to release iron from ferritin. The ability of phosphine to promote the release of iron from ferritin should not be surprising. Encouraged by the difference in redox potential between phosphine (− 1180 mV) and ferric iron (Fe^{3+})

(− 190 mV), it seems possible that phosphine could reduce and release stored iron from ferritin. Free ferrous iron (Fe^{2+}), once released from ferritin, will react with surrounding molecules. Under aerobic conditions, iron may react with molecular oxygen, resulting in oxoiron species such as perferryl ion (Fe^{2+}-O_2) and ferryl ion (Fe^{2+}-O) which are potent initiators of lipid peroxidation (Qian and Buettner, 1999). In addition, iron can generate hydroxy radical *via* the Fenton reaction, providing a potent initiator of lipid peroxidation.

Alteration of iron homeostasis by phosphine has not been suggested previously. We have now demonstrated that not only is phosphine capable of inducing iron release from ferritin *in vitro* but that the gene encoding ferritin-1 is induced in *C. elegans* in response to phosphine exposure as well. Since the ferritin gene in *C. elegans* is known to be regulated by iron (Gourley *et al.*, 2003) induction of the ferritin gene by phosphine is an indication that an increase in the cellular iron level has occurred. This suggests that phosphine toxicity may be caused or exacerbated by a disruption of iron homeostasis.

Ferritin plays a major role in iron homeostasis (Harrison and Arosio, 1996). There are two ferritin homologues in *C. elegans* designated *ftn-1* and *ftn-2*. The *ftn-2* gene shows a much higher basal levels of expression than the *ftn-1*, but the *ftn-1* gene is strongly induced by phosphine treatment. In mammals, the H and L subunit composition of ferritin varies between tissues. The L-rich ferritins found in iron storage organs such as liver and spleen generally contain more iron than the H-rich ferritins found in heart and brain (Harrison and Arosio, 1996). Despite the fact that both *C. elegans* ferritins are of the H type, it is possible that the two *C. elegans* isoforms exhibit different iron storage characteristics, analogous to what is observed in mammals.

In situ hybridization indicated that both genes are expressed in the digestive tract where iron is known to be absorbed from the diet. The highest expression of these genes is found in the proximal end of the alimentary tract where iron influx is highest. High-level expression is also found at the distal end of the alimentary tract, which is probably related to nutrient resorption prior to excretion of waste products. The tissues in which we observed ferritin gene expression were previously demonstrated to be sites of accumulation of a GFP fusion protein (Kim *et al.*, 2004), though that report also described some protein accumulation in other regions. The minor discrepancies in reported ferritin distribution between this report and that of Kim *et al.* (2004) are likely due to technical differences between direct determination of transcript abundance by *in situ* hybridization and indirect determination of gene expression topology through monitoring of accumulation of a chimeric transgene. This may have differed from normal transcript distribution as determined by *in situ* hybridization due to anomalous expression of the chimeric transgene, stability of the GFP protein, or simply the amplification of signal inherent in measuring protein levels as opposed to mRNA.

Quantitative real-time PCR shows that *ftn-1* gene but not *ftn-2* is induced by phosphine in a dose-dependent manner. The mechanism of *ftn-1* gene induction by phosphine in *C. elegans* is not known. It may be either by an iron-dependent or an iron-independent pathway since ferritin genes in other organisms are transcriptionally regulated by both oxidants and iron (Wilkinson *et al.*, 2003). However, the pattern of ferritin gene expression in response to phosphine in *C. elegans* is similar to the ferritin response to iron in that the *ftn*-1 gene, but not the *ftn*-2 gene, is induced (Gourley *et al.*, 2003; Kim *et al.*, 2004).

If phosphine causes an increase in the free iron level, regardless of where that iron originates, suppression of the ferritin gene expression should reduce the titer of protective ferritin protein, thereby increasing susceptibility to phosphine. Enhanced sensitivity to phosphine was indeed observed, but only when RNAi was used to suppress the gene encoding ferritin-2. Because ferritin-2 is the more highly expressed of the two ferritin genes, the observed 90% suppression of *ftn-2* expression seems to have disrupted iron homeostasis in the nematodes to a level that was not compensated by *ftn-1* induction. It seems that release of iron from ferritin by phosphine, together with a reduction in ferritin gene expression results in reduced capacity for iron sequestration and, therefore, susceptibility to iron overload. It is clear from even casual observation, however, that the magnitude of the change in gene suppression is not matched by a similar magnitude increase in sensitivity to phosphine. A possible explanation for this is that ferritin typically has significant unused iron storage capacity as would be expected of a structure designed to sequester iron to limit the concentration of free iron in the cell.

We have carried out the converse experiment and have shown that normal ferritin levels in a high iron environment result in increased sensitivity to phosphine in *C. elegans*. Since exposure to very high levels of iron does not affect the growth and development of the nematodes (Kim *et al.*, 2004), the resulting iron toxicity seems to result directly from the action of phosphine. Presumably, a threshold, beyond which *C. elegans* is no longer able to cope with high levels of free iron is reached.

Interestingly, the biochemical and histochemical responses to phosphine resemble those of iron overload. For example, the increase in lipid peroxidation observed in phosphine-treated cell lines (Hsu *et al.*, 1998), insects (Chaudhry and Price, 1992), rats (Hsu *et al.*, 2000, 2002a,b), and mice (Quistad *et al.*, 2000) is similar to that of iron-loaded $HepG_2$ cells (Jagetia *et al.*, 2004), as well as hepatic cells of iron-loaded rats (Brown *et al.*, 1998) and mice (Bartfay *et al.*, 1999). Moreover, the decrease of GSH in phosphine-treated rats (Hsu *et al.*, 2000, 2002b) was found in iron-loaded $HepG_2$ cells as well (Jagetia *et al.*, 2004).

CONCLUSION

The chemical properties of phosphine leave open the possibility that it could have a multitude of toxic effects, including release of iron from ferritin. *In vitro* results support the possibility that phosphine can act indirectly *via* disruption

of iron homeostasis. *In vivo*, this is confirmed by iron-mediated induction of at least one of the ferritin genes, as well as hypersensitivity to iron in the presence of phosphine. Taken together, we proposed that phosphine does indeed release iron from ferritin causing lipid peroxidation and that the resulting cellular damage contributes to phosphine toxicity.

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