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Biochemical Mechanisms of Phosphine Action and Resistance

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Abstract: Global grain shortages highlight the strategic requirement for dependable storage of food reserves. While phosphine fumigation has been the mainstay of grain protection for many years, resistance in pest insects threatens the continued effectiveness of phosphine. Effective management of phosphine resistance requires an understanding of how phosphine acts as well as how organisms can overcome its action. Despite the importance of phosphine, its mode of action has not been determined. To resolve this problem, we have investigated the toxicology of phosphine in the model organism, *C. elegans*, which is ideal for laboratory studies. We see that mitochondrial activity is directly related to the effectiveness of phosphine, as mutation, chemical and physical treatments that reduced respiration cause resistance. In contrast, chemicals that activate mitochondrial electron transfer enhance phosphine toxicity, allowing resistance to be completely overcome. We have found that key metabolic regulatory pathways that determine food abundance or deprivation are likewise capable of enhancing sensitivity toward phosphine, even in otherwise resistant organisms, apparently through regulation of metabolic rate. Our studies reflect and extend previous work with insects that demonstrated a requirement for oxygen and a role for mitochondria in phosphine toxicity. These results will assist us in the development of strategies to monitor resistance or enhance the efficacy of phosphine fumigation.

Key words: Phosphine, mitochondria, oxygen toxicity, reactive oxygen species, *Caenorhabditis elegans*.

Introduction

Phosphine, hydrogen phosphide (PH_3), is a poisonous gas used for fumigation of stored commodities. Currently it is the only fumigant with worldwide registration (MBTOC 2006). Its continued and widespread use is due to its low cost, rapid diffusion through a grain mass, ease of generation and use, and lack of production of toxic by-products which leaves grain free of harmful residues (Chaudhry 1997).

Despite the importance of phosphine, the mechanism of its toxicity remains poorly understood. It has been demonstrated that phosphine toxicity is dependent on the presence of molecular oxygen in both insects and nematodes (Bond 1963; Cheng *et al.* 2003; Kashi 1981). Aerobic respiration is also shown to be inhibited in a variety of species following phosphine administration (Pimentel *et al.* 2007). It is hypothesized that complex IV of mitochondrial respiratory chain (MRC), *cytochrome c oxidase*, is the primary site of action of phosphine (Chefurka 1976; Nakakita 1976). The inhibition of this mitochondrial enzyme induces the elevated production of reactive oxygen species (ROS) which caused damage to DNA, lipids, and protein molecules (Salvioli *et al.* 2001).

Phosphine exposure, both *in vitro* and *in vivo*, results in lipid peroxidation, which is believed to be the consequence of superoxide(s) generated from mitochondria. Furthermore, this lipid oxidation process was enhanced by the presence of transition metal ions such as iron (Cha'on *et al.* 2007; Qian and Buettner 1999). It is therefore not surprising that glutathione (GSH), a cellular antioxidant that can protect cells against oxidative damage has been shown to protect cells against phosphine (Hsu *et al.* 1998; Quistad *et al.* 2000).

The mechanisms whereby insects become resistant to phosphine are even less well understood than the mode of action of phosphine. Resistance to phosphine was first reported in pest insects of stored products in 1977 (Champ and Dyte 1977). Recent studies reveal that the situation is getting worse, as extremely resistant pest insects have been reported from around the world (141 in China (Li *et al.* 1994); 380 in India (Rajendran and Narasimhan 1994); 600

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in Australia (Collins *et al.* 2002); > 770 in Japan (Hori and Kasaishi 2005); and 1160 in Brazil (Athie *et al.* 1998)). Increasing phosphine resistance forces grain handlers to increase the fumigation time and/or phosphine concentration, which increases occupational health risks and results in increased cost. Greater understating of the phosphine toxicity and resistance mechanisms will allow more effective management of pest insects and containment of the spread of resistance.

In order to gain more understanding, we initiated the investigation of phosphine action in the model organism, a nematode, *Caenorhabditis elegans*. *C. elegans* was selected due to its rapid lifecycle, small size, small genome, ease of culture, and availability of genetic resources. We have created a phosphine-resistant line (*pre* – 33) in *C. elegans*, which is 9 times more resistant to phosphine than is the wild-type strain (Cheng *et al.* 2003) and as resistant to phosphine as most highly resistant insect lines.

Phosphine Toxicity Requires Oxygen

One of the first tasks in developing *C. elegans* as a model for the study of phosphine toxicity was to confirm that phosphine acts in *C. elegans* as it does in insects. A key feature of phosphine toxicity in insects is that oxygen not only enhances toxicity, but in atmospheres of less than 2% oxygen, phosphine is completely harmless (Bond 1963; Kashi 1981). As with insects, we found that oxygen enhances phosphine toxicity in *C. elegans* as wild-type animals showed a dose-dependent increase in mortality with increasing oxygen concentration even though the phosphine concentration was maintained at a low level (0.1mg/L) (Cheng *et al.* 2003). Very interestingly, the *pre* – 33 mutant line that was resistant toward phosphine was also completely resistant to the synergistic enhancement of phosphine toxicity by high oxygen levels (Fig 1). Even when the phosphine concentration was increased to 0.6mg/L, there was no significant increase in mortality. This result suggests that phosphine toxicity might be mediated through oxidative stress since it is dependent on the presence of oxygen.

Synchronized 42 h – old nematodes were treated with 0.1 mg/Lphosphine at 25 C for 24 h at the indicated concentrations of oxygen. The effect of hyperoxia on *pre* – 33 under 0.6mg/L phosphine is also shown as a dashed line. All results are the average of two experiments.

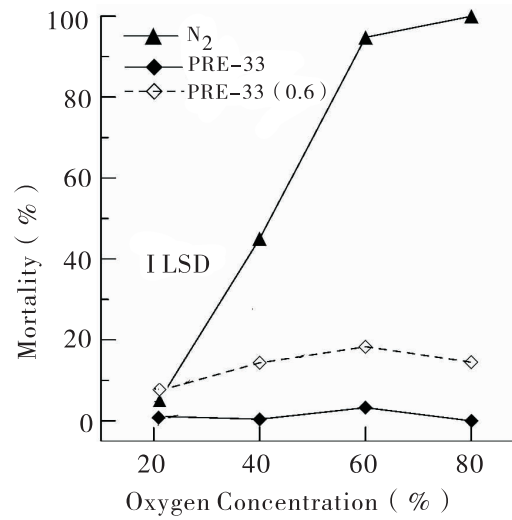

Fig. 1 The effect of oxygen concentration on phosphine – induced mortality in *C. elegans*

Differences between data points greater than the LSD bar on the left side of the figure are considered to be significant. Note 1 mg/L phosphine equals approximately 700ppm.

Phosphine Inhibits Mitochondrial Activity

Mitochondria are the proposed target of phosphine as demonstrated by *in vitro* studies of mitochondria isolated from pest insects (Chefurka 1976). We took advantage of *C. elegans* to test whether mitochondrial function is likewise disturbed by phosphine in live animals (Zuryn *et al.* 2008). We found that the respiration rate was inhibited by 70% within 1 hour of phosphine exposure in wild-type animals (Fig. 2). Paradoxically, the respiration rate of the *pre* – 33 mutant is much lower than in wild-type animals in the absence of phosphine exposure. Phosphine exposure, however, failed to lower the respiration rate any further than was observed for the wild type strain (Fig. 2). This presented the unusual situation that the metabolic disruption caused by phosphine exposure in wild type animals was also observed in mutant animals that were resistant to phosphine exposure. To resolve this apparent contradiction, we then measured the mitochondrial membrane potential in these animals. In the wild type strain, phosphine caused a decrease in mitochondrial membrane potential (MMP) after 5 hours as determined by fluorescence of the dye tetramethylrhodamine ethyl ester (TMRE; Sigma) (Fig. 3). TMRE is a cell permeable cationic dye which accumulates in intact mitochondria in proportion to the MMP (Ehrenberg *et al.* 1988). A similar decrease in MMP was ob-

served in mutant animals, but because they had a higher basal MMP, the net result was that membrane potential was preserved in these animals relative to wild type under equivalent exposure to phosphine (Fig. 3). Thus, the reduction in mitochondrial respiration in mutant animals was likely a side-effect of a metabolic change elsewhere that allowed them to survive despite reduced mitochondrial activity.

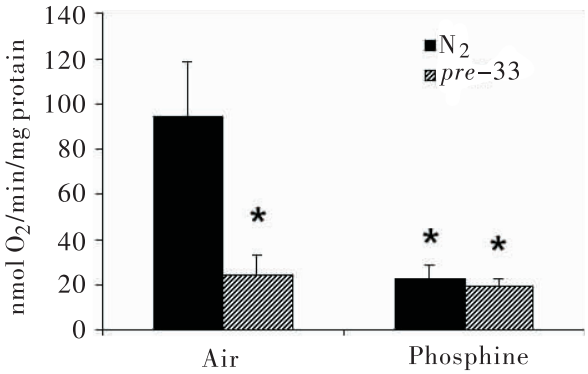

Fig. 2 Oxygen consumption rates in phosphine – treated nematodes.

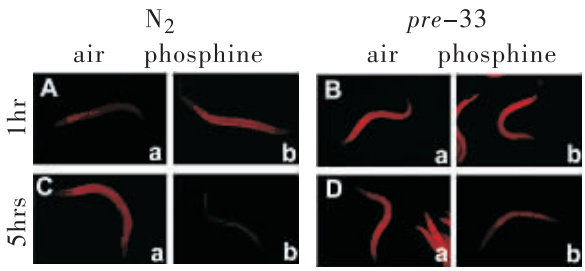

Fig. 3 Photographs of TMRE – stained nematodes exposed to phosphine.

The mutant phenotype strongly supports the metabolic theory of phosphine toxicity, by which factors that reduce the flow of electrons through the mitochondrial electron transport chain result in phosphine resistance, whereas factors that increase electron flow are proposed to enhance phosphine toxicity (Schlipalius *et al.* 2006). Preliminary work in our lab has indeed identified powerful phosphine synergists that completely overcome the resistance of *pre*-33 (unpublished). Another interesting point in the study of Zuryn *et al.* is that maintenance of the MMP despite reduced electron flow through the mitochondrial electron transport chain seems to be critical for the survival of phosphine resistant nematodes. Thus, the apparent paradox referred to above can be explained by proposing two distinct mechanisms of phosphine toxicity, the first of which is oxidative stress resulting from the interaction between phosphine and a high rate of electron flow through the mitochondrial electron transport chain, and the second of which is phosphine-induced collapse of the mitochondrial membrane potential. Collapse of the membrane potential is known in other organisms to trigger cellular suicide (apoptosis). In a multi-cellular organism, this serves to sacrifice individual cells that are metabolically defective in an effort to preserve other cells of the organism. In the case of phosphine exposure, the apoptotic response would be triggered in every cell of the organism, resulting in cataclysmic cell death.

Exposure to phosphine at 20℃ for 1 h reduces the respiration rate in N_2 nematodes *in vivo*. Phosphine resistant mutant *pre*-33 have lower respiration rates than normal and are therefore less affected by the phosphine exposure. Phosphine treatment was 70 ppm (sublethal for 24 h) on 48 h – old nematodes. $^{*}p < 0.05$ is significant differences compared with N_2 animals in air. There was no significant difference between *pre*-33 nematodes exposed to air alone and *pre*-33 nematodes exposed to phosphine. Columns represent means of three independent experiments with error bars representing the SEM. Note 1 mg/L phosphine equals approximately 700ppm.

The dye TMRE was used to qualitatively assess the mitochondrial membrane potential (MMP) of nematodes exposed to 350ppm phosphine at 20℃. Wild type (N_2) animals (A, C) exposed to phosphine (b) had a much lower MMP than their counterparts in air (a). This was a similar scenario to what was observed in *pre*-33 mutant nematodes (B, D), which are resistant to phosphine-induced mortality. Note that *pre*-33 nematodes have a higher basal MMP compared with N_2 nematodes both in air and following phosphine exposure in most cases. Note 1 mg/L phosphine equals approximately 700ppm.

Phosphine and Diethyl Maleate Synergism

Glutathione (GSH) is a cellular antioxidant compound that protects against oxidative stress. Valmas and Ebert (2006) first reported the synergistic effect of phosphine and the GSH depleting compound diethyl maleate, clearly implicating oxidative stress in phosphine toxicity in *C. elegans*. The LD_{50} of phosphine and diethyl maleate co-treatment is doubled compared to diethyl maleate alone (Fig. 4). It has been reported phosphine is able to inhibit the activity of the antioxidant enzymes, catalase and peroxi-

dase in both resistant and sensitive insects (Chaudhry and Price 1992). Such a situation would leave GSH as one of the primary remaining defences against phosphine-induced oxidative stress. This finding supports the hypothesis that phosphine exposure results in lethal oxidative damage. Phosphine has actually been shown to exacerbate the problem of mitochondrially-produced reactive oxidants because phosphine and hydrogen peroxide can interact to form the much more highly reactive hydroxyl radical that readily reacts with lipids and other key molecules in the cell (Quistad *et al.* 2000).

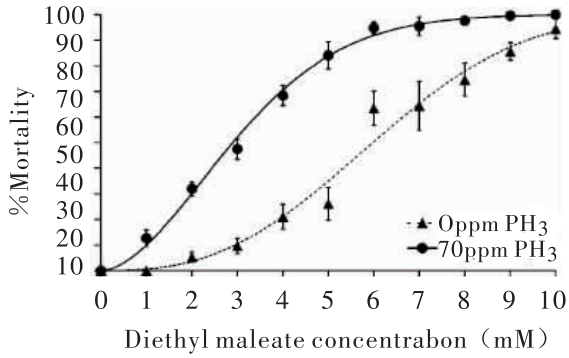

Fig. 4 Diethyl Maleate Interaction with Phosphine.

Mortality of wild-type (N_2) *C. elegans* when exposed to diethyl maleate and two different doses of phosphine: 0 ppm (▲); and 70 ppm (·); at 20℃ for 24 h. Regressions lines are based on complementary log – log/log relationships, and data points are weighted means from biological replicates ± weighted SEM. The LC50 of DEM in the absence of phosphine at 20℃ for N2 is 5.98 mM; and with 70 ppm PH_3 is 2.9 mM. All plates were counted after 24 h recovery. Note 1 mg/L phosphine equals approximately 700ppm.

Disruption of Iron Homeostasis Incre Ases Phosphine Toxicity

Metabolic disruption is not the only possible mechanism of action of phosphine, because phosphine also interacts strongly with specific metal ions, such as iron which are essential for the activity of a large number of cellular enzymes. Because iron is toxic when it is free in the cell as opposed to bound up in enzymes, a special storage protein, ferritin, ensures that iron is available while preventing it from doing harm. We supposed that phosphine-mediated release of iron from the ferritin store might contribute to the toxicity of phosphine. This hypothesis is supported by phosphine-mediated induction of ferritin gene expression. Fig. 5 clearly shows the dose-dependent induction of ferritin mRNA in response to phosphine exposure (Cha'on *et al.* 2007).

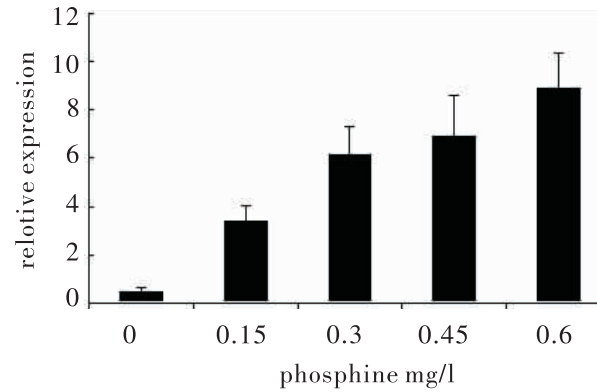

Fig. 5 Ferritin – 1 induction by phosphine.

42 h old adults N_2 nematodes were exposed to phosphine at different concentrations (0, 0.15, 0.3, 0.45 and 0.6 mg/L) for 10 h at 20℃. The level of ferritin – 1 mRNA is analysed by quantitative real-time PCR. Columns are means of five biological replicates. Error bars are SEM. Note 1 mg/L phosphine equals approximately 700ppm.

Furthermore, *C. elegans* is hypersensitive to phosphine under conditions of iron overload (Fig 6). Whereas an increase in iron concentration in the absence of phosphine caused no significant increase in mortality, the same iron concentrations resulted in dose-dependent mortality in the presence of an otherwise sub-lethal dose of phosphine. Finally, the iron released from ferritin in response to phosphine exposure results in lipid peroxidation, a hallmark of phosphine toxicity (Cha'on *et al.* 2007).

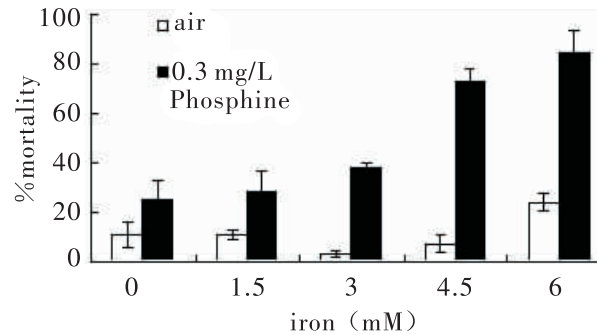

Fig. 6 Iron overload increased phosphine toxicity in *C. elegans*.

24h – aged N_2 nematodes were transferred to normal medium and iron-rich medium (1.5, 3, 4.5, 6 mM). Animals were left over night before exposed to either air or air with 0.3 mg/L phosphine for 24 hr at 20℃. The mor-

tality of those exposed to air was investigated immediately after 24 h exposure, whereas those under phosphine exposure were examined after a 24 h recovery period. Columns are means of three biological replicates. Error bars are SEM. Note 1 mg/L phosphine equals approximately 700ppm.

Conclusion

The mode of action of phosphine toxicity has been unclear and the mechanism of phosphine resistance is even less well studied. We have confirmed and extended our understanding of the biochemical mechanisms using the model organism *C. elegans*, in combination with genetic analysis of phosphine resistance in both insects and *C. elegans*. We have confirmed that phosphine toxicity is dependent on the availability of molecular oxygen and that the toxicity is elevated in response to increasing concentrations of oxygen as is consistent with the oxidative stress model of phosphine toxicity. We also demonstrated that mitochondrial function is disrupted by phosphine and that a key distinction between sensitive and resistant animals is the ability of the latter to maintain mitochondrial membrane potential in the face of phosphine exposure. Compounds that greatly enhance the toxicity of phosphine were identified that exploit each of these toxicity mechanisms. We also found that iron homeostasis has an impact on phosphine toxicity. Even though the influence of iron release on phosphine toxicity was much smaller than the other two mechanisms, the existence of high level genetic resistance among pest insects indicates that the primary mechanisms of phosphine action have been overcome. It is entirely likely that we now rely on secondary modes of action of phosphine for pest control in the field. Thus, understanding both primary and secondary mechanisms of phosphine action will be essential if we are to maintain the effectiveness of phosphine for years to come.

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