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磷化氫在體內所引起氧化性毒害之探討
Phsophine-Induced Oxidative Damage In Vivo

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一、中文摘要

磷化氫是一種高毒性氣體。它是一廣泛使用之工業物品，其主要用途包括在半導體製造上做為摻雜劑及農產品薰蒸劑、滅鼠劑（可經由磷化鋁、磷化鎂或磷化鋅潮解而產生）。由於鹵化碳氫化物類殺蟲劑及甲基溴的限用，再在加上半導體產品的普及化，未來預期會提高磷化氫之使用量。以往相關研究顯示磷化氫可造成昆蟲、哺乳動物及哺乳動物細胞株之氧化性傷害。

本研究探討磷化氫對大白鼠造成氧化性傷害及使用抗氧化劑保護之情形。Wistar 雄性大白鼠以腹腔注射 2 mg/kg 的磷化氫，30 分鐘後，將大白鼠殺死取出腦、肝及肺，分析其麩胺基硫及脂質過氧化產物變化情形，並於腦及肝中觀察其 8-hydroxydeoxyguanosine (8-OH-dGuo) 之含量。結果發現磷化氫明顯下降麩胺基硫之濃度、0 明顯增加脂質過氧化：腦部改變值為 36-42%，肺改變值為 32-38%，肝改變值為 19-25%；腦及肝中的 8-OH-dGuo 亦顯著增加：腦部增加 70%、肝則增加 39%。

另一組大白鼠則於注射磷化氫 30 分鐘前，先投予抗氧化劑：褪黑激素 10mg/kg、維生素 C 30mg/kg 及 β-胡蘿蔔素 6mg/kg。結果發現磷化氫所引起氧化性傷害被褪黑激素明顯或完全抑制，維生素 C 及 β-胡蘿蔔素則活性較弱甚至無作用。本研究證實磷化氫造成大白鼠的腦、肝及肺氧化傷害，褪黑激素對其具相當之保護功能，此外易反應之含氧物種在磷化氫之基因毒性上扮演了相當重要的角色。

關鍵詞：褪黑激素、抗氧化劑、農藥、磷化氫、自由基、麩胺基硫、脂質過氧化

Abstract

Phosphine (PH_3), from hydrolysis of aluminum, magnesium and zinc phosphide, is an insecticide and rodenticide. Earlier observations on PH_3-poisoned insects, mammalian cell lines and humans led to the proposed involvement of oxidative damage in the toxic mechanism. This investigation focused on PH_3-induced oxidative damage in rats and antioxidants as candidate protective agents. Male Wistar rats were treated ip with PH_3 at 2 mg/kg. Thirty min later the brain, liver, and lung were analyzed for glutathione (GSH) levels and lipid peroxidation (as malondialdehyde and 4-hydroxyalkenals) and brain and lung for 8-hydroxydeoxyguanosine (8-OH-dG). PH_3 caused a significant decrease in GSH concentration and elevation in lipid peroxidation in brain (36-42%), lung (32-38%) and liver (19-25%) and significant increase in 8-OH-dG in brain (70%) and liver (39%). Antioxidants administered ip 30 min before PH_3 were melatonin, vitamin C and β-carotene at 10, 30 and 6 mg/kg, respectively. The PH_3-induced changes were significantly or completely blocked by melatonin while vitamin C and β-carotene were less effective or inactive. These findings establish that PH_3 induces and melatonin protects against oxidative damage in the brain, lung and liver of rats and suggest the involvement of reactive oxygen species in the genotoxicity of PH_3.

Keywords:Melatonin, free radicals, pesticide, lipid peroxidation, 8-hydroxydeoxyguanosine phosphine, glutathione, antioxidants

二、緣由與目的

Phosphine (PH_3) is a widely-used fumigant for the control of stored product insects. It is normally generated by the action of ambient water vapor on a solid formulation containing aluminium or magnesium phosphide mixed with other

ingredients designed to regulate the release of the gas. At present the major fumigants for controlling insect pests in stored products are PH_3 and methyl bromide. Since methyl bromide is being phased out because of adverse environmental effects, the role of PH_3 is becoming of even greater importance [1]. Additionally, zinc phosphide is a major rodenticide, liberating PH_3 when ingested [2]. PH_3 is also used in the synthesis of organophosphines and as a dopant in semiconductor production [3].

PH_3 is highly toxic to many animals [3]. AlP, with a rat oral LD_{50} of 14 mg/kg [4], is responsible for many human poisonings in India [5,6]. Fumigators exposed to PH_3 may have an increased frequency of chromosomal abberations in their peripheral blood lymphocytes [7-9]. PH_3 is weakly genotoxic in mice, i.e., exposure of Balb-c mice (4.5 ppm, 13 weeks) results in significant increases in micronucleus frequency in bone marrow and spleen lymphocytes [10].

PH_3 is a respiratory inhibitor and induces oxidative damage in animals [11]. It inhibits the activities of cytochrome c oxidase [12-14], catalase [15-18] and peroxidase [16], stimulates the production of hydrogen peroxide and reactive oxygen species (ROS) [14,19] and elevates superoxide dismutase (SOD) [16,17]. The malondialdehyde (MDA) level is elevated in cardiac tissue of AlP-poisoned rats [20]. Consistent with these observations, AlP-poisoned humans show significantly higher SOD and MDA levels and lower catalase levels in serum compared to unexposed patients [5]. Intravenous magnesium reduces oxidative stress and mortality in humans with acute AlP poisoning [6].

Melatonin, a major secretory product of the pineal gland, scavenges hydroxyl radical [21], peroxynitrite [22], singlet oxygen [23], and possibly peroxyl radical [24] which is generated during the oxidation of unsaturated lipids and leads to the propagation of lipid peroxidation. The effectiveness of melatonin is facilitated by its combined lipophilic [25] and hydrophilic [26] character, allowing transport across the blood-brain-barrier and distribution throughout the cell [27,28]. Melatonin may reduce oxidative stress also by stimulating some important antioxidative enzymes, i.e., SOD [29], GSH reductase [30], glucose-6-phosphate dehydrogenase [31], and glutathione (GSH) peroxidase, perhaps the most important antioxidant enzyme in brain [32].

PH_3 causes oxidative toxicity in insects, mammalian cells, rats and humans. However, there are no reports on the effect of antioxidants on PH_3-induced oxidative stress in mammals. The first aim of the present study is to examine possible PH_3-induced oxidative damage in rats using as criteria the levels of GSH, glutathione disulfide (GSSG), lipid peroxidation products (MDA plus 4-hydroxyalkenals (4-HDA)), and 8-hydrodeoxyguanosine (8-OH-dGuo) in DNA. The second goal is to compare melatonin with vitamin C and β-carotene as candidate antioxidants to protect against oxidative damage induced by PH_3.

三、研究報告(結果與討論)

PH_3 adminstered ip at 2 mg/kg induced lipid peroxidation (measured as MDA plus 4-HDA) in brain within 15 min, i.e., 123 ± 13% (n=6) relative to control values. The level increased further at 30 min (142 ± 9%) and remained unchanged at 90 min (148 ± 10%). These observations led to a more detailed study of PH_3-induced oxidative damage and standardization of the conditions with sacrifice 30 min after treatment with PH_3.

PH_3 significantly decreased GSH and increased GSSG levels in all test tissues (Table 1). The antioxidants in themselves did not affect the GSH and GSSG levels. Melatonin pretreatment completely abolished PH_3-induced changes in GSH and GSSG concentrations in brain and liver and ameliorated the effect in lung; in each case the reversal was statistically significant. Vitamin C reduced the PH_3-induced GSH and GSSG changes, though not to a

significant degree when compared to the PH_3-treated animals. β-Carotene had little or no effect on PH_3-induced changes.

PH_3 significantly increased lipid peroxidation in brain, lung, and liver above the levels in control animals (Table 1). Lipid peroxidation was not affected by the antioxidants alone. The PH_3-induced increase was significantly or completely blocked by melatonin pretreatment in all assayed tissues. In contrast, vitamin C and β-carotene did not significantly attenuate the PH_3-induced increase in brain, lung, and liver.

PH_3-induced DNA damage was evident in brain and liver by the higher 8-OH-dGuo/dGuo ratio than found in control animals (Table 1). Melatonin significantly reversed the PH_3-induced changes in brain and liver. Vitamin C marginally but not significantly diminished the elevated ratios. β-Carotene was the least effective in protecting against DNA damage.

四、計畫成果自評

This study establishes that PH_3 induces oxidative damage in brain, lung, and liver of rats with partial to complete protection by melatonin and less attenuation by vitamin C and β-carotene (Table 2). More specifically, PH_3 significantly decreases GSH and increases GSSG concentrations in brain, lung and liver (36-44%, 34-38%, and 18-19%, respectively); these changes are attenuated or negated by melatonin, with much less effect of vitamin C or β-carotene. These findings on PH_3 and antioxidant effects are closely paralleled by the elevation in lipid peroxidation by 42%, 32% and 25% in brain, lung, and liver, respectively, in each case largely or completely abolished by melatonin and partially by vitamin C and β-carotene. The 8-OH-dGuo/dGuo ratios are also markedly elevated by PH_3 in brain and lung (70% and 39%, respectively) with significant protection by melatonin but not by vitamin C and β-carotene. On an overall basis, the antioxidants reduce PH_3-induced oxidative damage with an effectiveness order of melatonin > vitamin C $\geqq$ β-carotene.

The action of PH_3 in rats reported here correlates well with our previous findings of increased H_2O_2 production, lipid peroxidation and oxidized DNA in Hepa 1c1c7 cells [19]. Decreased GSH in rat tissues with a concurrent rise in GSSG strongly suggests the involvement of ROS in PH_3 toxicity. Depletion of GSH favors lipid peroxidation and predisposes cells to oxidant damage [33]. The failure to observe PH_3-induced changes in GSH levels in cultured cells [19] and insects [14,16] may relate to GSH resynthesis with the long experimental periods involved. The toxicity of PH_3 is dependent on the presence of oxygen [15]. PH_3 induces a higher degree of oxidative toxicity in brain and lung than in liver possibly associated with the higher oxygen exchange and unsaturated lipid content with the first two tissues [34].

Melatonin, the most effective antioxidant under the test conditions, limits GSH depletion, GSSG formation, lipid peroxidation, and 8-OH-dGuo formation in tissues of PH_3-treated rats. These findings are consistent with previous studies where melatonin protects against oxidative damage of other ROS-generating agents, e.g. paraquat, cyanide, and kainic acid, and is a potent scavenger of hydroxyl and peroxyl radical both *in vivo* and *in vitro* [21,24,35-37].

In conclusion, the overall findings establish that PH_3 induces and melatonin protects against oxidative damage in the brain, lung and liver of rats and suggest the involvement of ROS in the genotoxicity of PH_3.

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Table 1. Effects of PH_3 and antioxidants on levels of GSH, GSSG, lipid peroxidation products and 8-OH-dGuo/dGuo ratio in ip-treated rats

Tissue	Antioxidant Pretreatment			
Toxicant	None	Melatonin	Vitamin C	β-Carotene
GSH level (and GSSG level in parenthesis), μmol/g tissue				
Brain				
Control	1.45 ± 0.11 (0.018 ± 0.001)	1.53 ± 0.11 (0.017 ± 0.002)	1.48 ± 0.07 (0.018 ± 0.001)	1.52 ± 0.13 (0. 018 ± 0.001)
PH_3	0.93 ± 0.11[a] (0.026 ± 0.002[a])	1.53 ± 0.09[b] (0.017 ± 0.001[b])	1.13 ± 0.10[a] (0.022 ± 0.001[a])	0.98 ± 0.10[a] (0.023 ± 0.002[a])
Lung				
Control	1.30 ± 0.20 (0.038 ± 0.002)	1.33 ± 0.16 (0.036 ± 0.002)	1.30 ± 0.10 (0.037 ± 0.002)	1.33 ± 0.11 (0.037 ± 0.001)
PH_3	0.80 ± 0.09[a] (0.051 ± 0.003[a])	1.20 ± 0.07[b] (0.036 ± 0.002[b])	1.08 ± 0.09 (0.043 ± 0.002)	1.00 ± 0.10 (0.045 ± 0.003[a])
Liver				
Control	4.40 ± 0.18 (0.110 ± 0.004)	4.38 ± 0.22 (0.108 ± 0.003)	4.42 ± 0.11 (0.112 ± 0.003)	4.38 ± 0.12 (0.109 ± 0.005)
PH_3	3.58 ± 0.20[a] (0.130 ± 0.003[a])	4.40 ± 0.09[b] (0.114 ± 0.006[b])	4.00 ± 0.18 (0.119 ± 0.004)	3.93 ± 0.12[a] (0.122 ± 0.004)
MDA + 4-HDA level, nmol/mg protein				
Brain				
Control	3.00 ± 0.07	2.80 ± 0.04[c]	2.99 ± 0.10	3.03 ± 0.11
PH_3	4.27 ± 0.10[a]	3.10 ± 0.09[ab]	4.01 ± 0.20[a]	4.12 ± 0.13[a]
Lung				
Control	2.58 ± 0.09	2.57 ± 0.17	2.58 ± 0.14	2.68 ± 0.14
PH_3	3.40 ± 0.20[a]	2.58 ± 0.09[b]	3.01 ± 0.18	3.21 ± 0.17[a]

Liver				
Control	0.81 ± 0.02	0.78 ± 0.06	0.80 ± 0.03	0.90 ± 0.04
PH_3	1.01 ± 0.05[a]	0.80 ± 0.03[b]	0.80 ± 0.10	0.89 ± 0.14

(Table 1 continued)

8-OH-dGuo/10^5 dGuo ratio				
Brain				
Control	3.18 ± 0.25	2.72 ± 0.21	3.20 ± 0.28	3.10 ± 0.36
PH_3	5.40 ± 0.47[a]	3.02 ± 0.37[ab]	4.48 ± 0.40[a]	5.02 ± 0.32[a]
Liver				
Control	4.30 ± 0.37	3.53 ± 0.39	4.25 ± 0.40	4.12 ± 0.44
PH_3	5.98 ± 0.63[a]	4.00 ± 0.32[b]	4.67 ± 0.50	5.50 ± 0.46

Rats were treated ip with PH_3 and tissues analyzed 30 min later. Antioxidant pretreatment was 30 min before PH_3. For conditions see Materials and Methods. The tabulated results in the left column are for pretreatments with saline but the findings with 3% ethanol or corn oil are not significantly different and in fact are practically identical (data not given). Data are mean ∀ SEM, n=6.

[a] PH_3 significantly different from corresponding control without PH_3 ($p < 0.05$).

[b] Melatonin with PH_3 significantly different from no antioxidant with PH_3 ($p < 0.05$).

[c] Melatonin significantly lower than the corresponding control without antioxidant ($p < 0.05$).

Table 2. Percentage effects of PH_3 alone and with antioxidants on GSH, GSSG, lipid peroxidation products and 8-OH-dGuo/dGuo ratio in ip-treated rats (PH_3/corresponding control x 100)

ıalyte	Antioxidant Pretreatment			
Tissue	None	Melatonin	Vitamin C	β-Carotene
;SH				
Brain	64%[a]	100%[b]	76%[a]	64%[a]
Lung	62%[a]	92%[b]	83%	75%
Liver	81%[a]	100%[b]	90%	90%[a]
SSG				
Brain	144%[a]	100%[b]	122%[a]	128%[a]
Lung	134%[a]	100%[b]	116%	122%[a]
Liver	118%[a]	106%[b]	106%	112%
DA + 4-HAD				
Brain	142%[a]	[bc]	134%[a]	136%[a]
Lung	132%[a]	100%[b]	117%	120%[a]
Liver	125%[a]	103%[b]	100%	99%
DH-dGuo/dGuo				
Brain	170%[a]	[bc]	140%[a]	162%[a]
Liver	139%[a]	113%[b]	110%	133%

Data from Table 1

[a] PH_3 significantly different from corresponding control without PH_3 ($p < 0.05$).

[b] Melatonin with PH_3 significantly different from no antioxidant with PH_3 ($p < 0.05$).

[c] Melatonin significantly lower than the corresponding control without antioxidant ($p <$ 0.05). The significant protective effect of melatonin to near control levels is partially obscured by its direct inhibitory action (see Table 1).