Free Radical Biology & Medicine, Vol. 28, No. 4, pp. 636–642, 2000

0891-5849/00/$–see front matter

PII S0891-5849(99)00277-4

Original Contribution

PHOSPHINE-INDUCED OXIDATIVE DAMAGE IN RATS: ATTENUATION BY MELATONIN

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(*Received* 8 *September* 1999; *Accepted* 14 *December* 1999)

Abstract—Phosphine (PH_3), from hydrolysis of aluminum, magnesium and zinc phosphide, is an insecticide and rodenticide. Earlier observations on PH_3-poisoned insects, mammals and a mammalian cell line 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-dGuo) in DNA. 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 of 8-OH-dGuo in DNA of 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. © 2000 Elsevier Science Inc.

Keywords—Melatonin, Antioxidants, Pesticide, Phosphine, Free radicals, Glutathione, Lipid peroxidation, 8-Hydroxydeoxyguanosine

INTRODUCTION

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. Because 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 [15–17] and peroxidase [15,16], stimulates the production of hydrogen peroxide and reactive oxygen species (ROS) [14,18] and elevates superoxide dismutase (SOD) [15,16]. The malondialdehyde (MDA) level is elevated in cardiac tissue of AlP-poisoned rats [19]. Consistent with these obser-

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vations, AlP-poisoned humans show significantly higher SOD and MDA levels and lower catalase levels in serum compared with unexposed patients [5]. Intravenous magnesium reportedly reduces oxidative stress and mortality in humans with acute AlP poisoning [6].

Melatonin, a major secretory product of the pineal gland, scavenges hydroxyl radical [20], peroxynitrite [21], singlet oxygen [22], and possibly peroxyl radical [23], which are generated during the oxidation of unsaturated lipids and lead to the propagation of lipid peroxidation. The effectiveness of melatonin is facilitated by its combined lipophilic [24] and hydrophilic [25] character, allowing transport across the blood–brain-barrier and distribution throughout the cell [26]. Melatonin may reduce oxidative stress also by stimulating some important antioxidative enzymes, i.e., SOD [27], glutathione (GSH) reductase [28], glucose-6-phosphate dehydrogenase [29], and GSH peroxidase [30].

PH_3 causes oxidative toxicity in insects, mammals, and mammalian cell lines. 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, GSH disulfide (GSSG), lipid peroxidation products (MDA plus 4-hydroxyalkenals (4-HDA)), and 8-hydroxydeoxyguanosine (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.

MATERIALS AND METHODS

Chemicals and treatment solutions

AlP (> 95% purity) was from Chem Service (West Chester, PA, USA) and other compounds from Sigma Chemical (St. Louis, MO, USA). A saturated aqueous solution of PH_3 was prepared by introducing freshly-powdered AlP (20 mg) into a 30 ml serum vial that was then stoppered with a rubber septum, and saline (20 ml) was injected in through the septum with immediate mixing. Insoluble salts were allowed to precipitate for 2 min before taking up the liquid portion (with some suspended solid) into a hypodermic syringe. This preparation is nominally 1 mg AlP/ml or 0.59 mg PH_3 equivalents/ml. However, some of the PH_3 would be in the gas phase and the solubility of PH_3 in water is 11.6 mM [11] or 0.39 mg PH_3/ml, which was considered to be the actual concentration. The PH_3 solution was administered ip to rats at 0.5 ml/100 g, equivalent to 2 mg PH_3/kg body weight. Melatonin was dissolved in ethanol then saline added to 3% final ethanol concentration. Vitamin C and β-carotene were dissolved in saline and corn oil, respectively.

Table 1. Three Sets of Experiments With Different Antioxidants (1–3), Each One Involving 24 Male Wistar Rats, Fasted Overnight, and Randomly Divided Into Four Groups of Six Rats (a–d)

Set	Antioxidant	Group	Carrier solution	Saline
1	melatonin	a	alone	alone
2	vitamin C	b	alone	PH_3
3	β-carotene	c	antioxidant	alone
		d	antioxidant	PH_3

The antioxidant solutions were administered ip at 0.5 ml/100 g body weight to yield dosages for melatonin, vitamin C, and β-carotene of 10, 30, and 6 mg/kg, respectively.

Experimental design (animal treatment)

Male Wistar rats (250 $\pm$ 30 g) were maintained on rat chow basal diet and water ad libitum. Three sets of experiments were made with different antioxidants (1–3), each one involving 24 rats fasted overnight and randomly divided into four groups of six rats (a–d) (Table 1).

Each rat received carrier solvent (saline, 3% ethanol, or corn oil) alone or with an antioxidant (vitamin C, melatonin, or β-carotene, respectively) followed after 30 min by saline alone or with PH_3. The findings with group a were the same in each of the three sets and in group b were also the same in each set, i.e., there was no significant difference in the controls for the three carrier solvents and in the effect of PH_3 following each of the carrier solvent pretreatments.

Rats killed by carbon dioxide anesthesia were subjected to intracardiac perfusion with ice-cold saline to eliminate excess iron that could otherwise be released from intracellular storage sites resulting in an artificial increase in free radical formation [31]. Brain, lung, and liver were removed and immediately frozen on solid carbon dioxide. All tissues were kept at -80°C until the time of the analysis.

Measurement of GSH and GSSG

A portion of each tissue was homogenized at 20% (w/v) in 1% picric acid in water using a glass-Teflon Potter-Elvehjem tissue grinder. The supernatant from centrifugation (15,000 $\times$ *g*, 15 min, 4°C) was used to measure GSH and GSSG by a procedure involving GSH reductase, 2-vinylpyridine and 5,5′-dithio-bis(2-nitrobenzoic acid) [32,33].

Measurement of aldehydic lipid peroxidation products

Tissue was homogenized as above at 10% (w/v) in ice-cold 20 mM Tris-HCl buffer, pH 7.4. The superna-

Table 2. 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	1.53 ± 0.11	1.48 ± 0.07	1.52 ± 0.13
	(0.018 ± 0.001)	(0.017 ± 0.002)	(0.018 ± 0.001)	(0.018 ± 0.001)
PH_3	0.93 ± 0.11[a]	1.53 ± 0.09[b]	1.13 ± 0.10[a]	0.98 ± 0.10[a]
	(0.026 ± 0.002[a])	(0.017 ± 0.001[b])	(0.022 ± 0.001[a])	(0.023 ± 0.002[a])
Lung				
Control	1.30 ± 0.20	1.33 ± 0.16	1.30 ± 0.10	1.33 ± 0.11
	(0.038 ± 0.002)	(0.036 ± 0.002)	(0.037 ± 0.002)	(0.037 ± 0.001)
PH_3	0.80 ± 0.09[a]	1.20 ± 0.07[b]	1.08 ± 0.09	1.00 ± 0.10
	(0.051 ± 0.003[a])	(0.036 ± 0.002[b])	(0.043 ± 0.002)	(0.045 ± 0.003[a])
Liver				
Control	4.40 ± 0.18	4.38 ± 0.22	4.42 ± 0.11	4.38 ± 0.12
	(0.110 ± 0.004)	(0.108 ± 0.003)	(0.112 ± 0.003)	(0.109 ± 0.005)
PH_3	3.58 ± 0.20[a]	4.40 ± 0.09[b]	4.00 ± 0.18	3.93 ± 0.12[a]
	(0.130 ± 0.003[a])	(0.114 ± 0.006[b])	(0.119 ± 0.004)	(0.122 ± 0.004)
MDA + 4-HDA level, nmol/mg protein				
Brain				
Control	3.00 ± 0.07	2.80 ± 0.09	2.99 ± 0.10	3.03 ± 0.11
PH_3	4.27 ± 0.10[a]	3.10 ± 0.09[b]	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
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[b]	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 at 2 mg/kg and tissues analyzed 30 min later. Antioxidant pretreatment (melatonin, vitamin C, or β-carotene at 10, 30, or 6 mg/kg, respectively) was 30 min before PH_3. For conditions see Materials and Methods. The tabulated results in the left column (none) 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 ($p < .05$).

[b] Melatonin with PH_3 significantly different from no antioxidant with PH_3 ($p < .05$).

tant from centrifugation (2500 × *g*, 30 min, 4°C) was analyzed for MDA plus 4-HDA using the lipid peroxidation kit of Calbiochem-Novabiochem Corp. (La Jolla, CA, USA) [18] as an estimate of lipid peroxidation [34]. The protein concentration was determined [35] with bovine serum albumin as the standard.

Measurement of 8-OH-dGuo/dGuo ratio

DNA was isolated from the brain and liver by treatment of the homogenate [10% (w/v) in 20 mM Tris-HCl buffer, pH 7.4] with 1% sodium dodecyl sulfate and 2.5 mg/ml proteinase K [36]. After extraction with phenol and then chloroform-isoamyl alcohol (24:1), DNA was precipitated by ethanol, dried, and enzymatically digested [37]. The nucleosides in a 20–50 μl aliquot were separated by HPLC (Hewlett Packard HP 1090, Palo Alto, CA, USA) on a Nucleosil C18 reverse-phase column (250 × 4.6 mm, 5 μm, Alltech Assoc. Inc., Deerfield, IL, USA) using 10% methanol in 50 mM potassium phosphate, pH 5.5, as the mobile phase at 1 ml/min for

Table 3. 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 ×100)

Analyte	Antioxidant pretreatment			
Tissue	None	Melatonin	Vitamin C	β-Carotene
GSH				
Brain	64[a]	100[b]	76[a]	64[a]
Lung	62[a]	92[b]	83	75
Liver	81[a]	100[b]	90	90[a]
GSSG				
Brain	144[a]	100[b]	122[a]	128[a]
Lung	134[a]	100[b]	116	122[a]
Liver	118[a]	106[b]	106	112
MDA + 4-HDA				
Brain	142[a]	111[b]	134[a]	136[a]
Lung	132[a]	100[b]	117	120[a]
Liver	125[a]	103[b]	100	99
8-OH-dGuo/dGuo				
Brain	170[a]	111[b]	140[a]	162[a]
Liver	139[a]	113[b]	110	133

Data from Table 2.
[a] PH_3 significantly different from corresponding control without PH_3 ($p < .05$).
[b] Melatonin with PH_3 significantly different from no antioxidant with PH_3 ($p < .05$).

30 min. The DNA content of 8-OH-dGuo was analyzed by electrochemical detection (LC-4B, Bioanalytical Systems, West Lafayette, ID, USA) and of deoxyguanosine (dGuo) by UV absorbance with a diode array detector [38]. Standard mixtures of 8-OH-dGuo and dGuo were also chromatographed to generate standard curves for quantitative analysis. The amount of 8-OH-dGuo was expressed as the number for every 10^5 dGuo in DNA.

Statistical analysis

Significant differences between multiple groups were determined using one-way ANOVA followed by the Bonferroni method. A probability of $p < .05$ was accepted as significant.

RESULTS

Preliminary study

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.

Effects of PH_3 and antioxidants on GSH and GSSG levels

PH_3 significantly decreased GSH and increased GSSG levels in all test tissues (Table 2). 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 without antioxidant. β-Carotene had little or no effect on PH_3-induced changes.

Effects of PH_3 and antioxidants on MDA + 4-HDA levels

PH_3 significantly increased lipid peroxidation in brain, lung, and liver above the levels in control animals (Table 2). 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.

Effects of PH_3 and antioxidants on 8-OH-dGuo/dGuo ratio

PH_3-induced DNA damage was evident in brain and liver by the higher 8-OH-dGuo/dGuo ratio found in treated than in control animals (Table 2). 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.

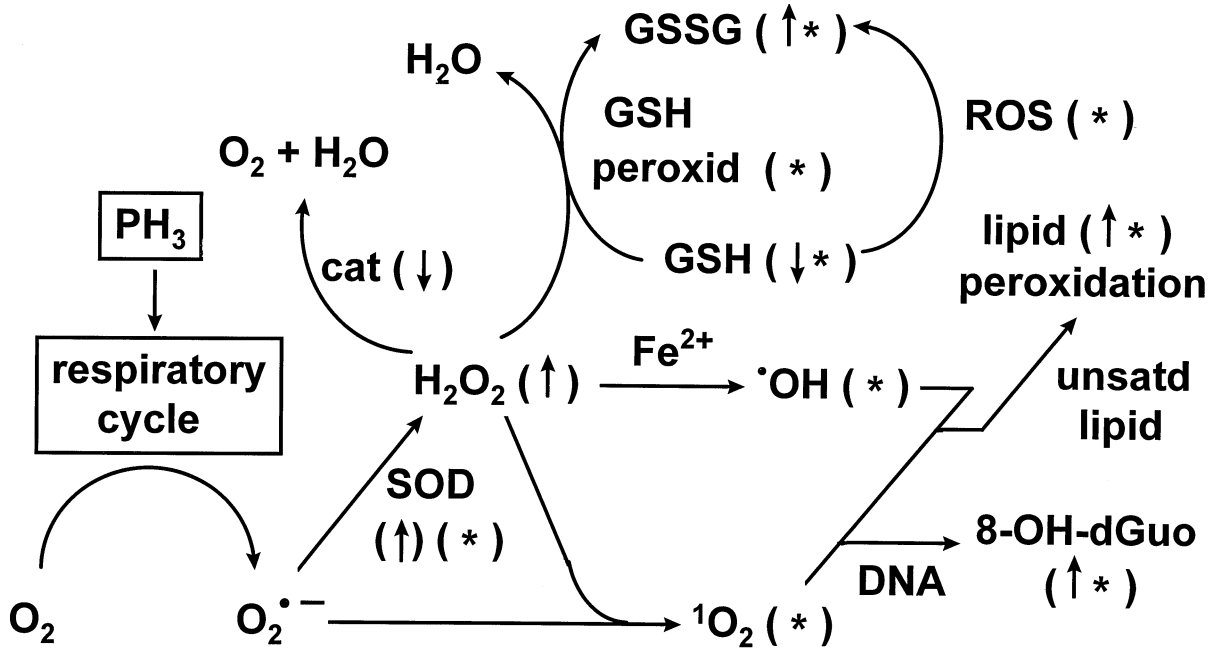

Fig. 1. Proposed scheme for PH_3-induced oxidative damage and alleviating action of melatonin. Arrows in parentheses designate PH_3-induced increase (up) or decrease (down) in activity or amount. Asterisk with an arrow indicates that melatonin attenuates or blocks the change induced by PH_3 (this study). Asterisk alone denotes that melatonin quenches ROS or stimulates an enzyme. The text provides further information on these relationships.

DISCUSSION

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 3). 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. The findings on PH_3 and antioxidant effects on GSH and GSSG levels 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 $\geq$ β-carotene.

GSH is a key component of the cellular defense cascade against injury caused by ROS and the level of GSH in tissues serves as an indicator of oxidative stress. GSH is a cofactor for GSH peroxidase, which catalyzes the reduction of hydrogen peroxide to water, thereby limiting the formation of hydroxyl radical, the most toxic of the oxygen-based radicals. 8-OH-dGuo is a sensitive biomarker of ROS-mediated DNA damage [39] and is considered to be an important lesion since it is mutagenic in DNA replication [40].

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 [18]. 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 [41]. The failure to observe PH_3-induced changes in GSH levels in cultured cells [18] and insects [14,15] may relate to GSH resynthesis with the long experimental periods involved. PH_3 induces a higher degree of oxidative toxicity in brain and lung than in liver possibly associated with their relative oxygen consumption and unsaturated lipid content [42].

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 radicals both in vivo and in vitro [20,23,43–45].

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 (Fig. 1).

Acknowledgements — The project described was supported by grants from the National Science Council, Executive Yuan, Republic of China (NSC 88-2314-B-038-148) and the United States National Institute of Environmental Health Sciences (grant RO1 ES08762), and its contents are solely the responsibility of the authors and do not necessarily represent the official views of the funding agencies. We thank Gary Quistad for helpful suggestions.

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ABBREVIATIONS

AlP—aluminium phosphide
dGuo—deoxyguanosine
GSH—glutathione
GSSG—glutathione disulfide
4-HDA—4-hydroxyalkenal (such as 4-hydroxy-2(E)-nonenal)
MDA—malondialdehyde
8-OH-dGuo—8-hydroxydeoxyguanosine
PH_3—phosphine
ROS—reactive oxygen species
SOD—superoxide dismutase