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PROTECTION OF RATS AGAINST THE EFFECTS OF α-NAPHTHYLTHIOUREA (ANTU) BY ELEVATION OF NON-PROTEIN SULPHYDRYL LEVELS*

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Abstract—We have investigated the influence of the elevation of pulmonary glutathione (GSH) levels on the toxicity of the rodenticide α-naphthylthiourea (ANTU) to rat lung. Administration of phorone (diisopropylidene acetone; 200 mg/kg i.p.) caused an initial depletion of both pulmonary and hepatic GSH followed after 48 hr by a marked elevation in both tissues, due most probably to a compensatory rebound synthesis. In control rats, ANTU produced a dose-dependent lethality, hydrothorax and loss of ability of lung tissue to accumulate adenosine and spermidine (markers of endothelial and epithelial cell function, respectively). These effects were prevented or markedly ameliorated when ANTU was given 48 hr after pretreatment with phorone. The mechanism of the protection by phorone pretreatment against ANTU-induced pulmonary toxicity is unclear. It may be due, in part, to elevated GSH levels in pulmonary endothelial cells and, in addition, to increased detoxification of ANTU in the liver, resulting in a decreased availability to the lung.

α-Naphthylthiourea (ANTU) is a rodenticide which produces a relatively selective pulmonary toxicity manifested by fibrin-rich non-haemorrhagic oedema and extensive pleural effusions in the rat and dog [1]. Pathological investigations of ANTU-induced injury suggested that oedema results from the reversible formation of gaps in the endothelium [2]. Meyrick *et al.* [3] showed a blebbing and scalloping of endothelial cells following ANTU administration.

Endothelial effects were also indicated by a reduction in 5-hydroxytryptamine uptake (a marker of endothelial function) observed in isolated perfused lungs prepared from rats dosed with ANTU [4]. Epithelial damage has also been observed, though this has been reported to occur subsequent to the endothelial injury [3]. The precise mechanism by which ANTU produces lung damage is uncertain [5]. Following *in vivo* administration of radiolabeled ANTU, covalent binding was observed to macromolecules in the lung and liver [6]. There is evidence that metabolic activation, involving desulphuration, occurs to form a reactive metabolite [6]. ANTU is metabolized, at least in part, by cytochrome P450 monooxygenase in both lung and liver microsomes to an intermediate which is capable of covalent binding [7]. Pretreatment *in vivo* with piperonyl butoxide (an inhibitor of cytochrome P450 monooxygenase activity) confers some protection against ANTU toxicity, supporting the involvement of metabolic activation [8].

Glutathione (GSH) is known to ameliorate the toxicities of various agents which damage the lung, including 3-methylindole [9], naphthalene [10] and 4-ipomeanol [11]. Previous studies have revealed that ANTU-induced lethality, hydrothorax and pulmonary covalent binding were potentiated by pretreatment with diethyl maleate (DEM), a potent GSH depleting agent [6]. Phorone (diisopropylidene acetone) is an α,β-unsaturated carbonyl compound which conjugates with GSH [12]. Phorone has been used in a variety of studies pertaining to the effects of GSH depletion on lipid peroxidation [13]. We have observed that after phorone administration, the initial GSH depletion is followed by an elevation to above control levels, which probably results from rebound synthesis. In the present study, we have investigated the influence of elevated pulmonary GSH levels on the toxicity of ANTU in the rat. We have also utilized functional markers of endothelial and epithelial cell integrity (adenosine and spermidine uptake, respectively) in order to assess ANTU-induced toxicity.

Recently, a number of studies have tried to quantify cellular damage to the lung by measuring the effect of lung damaging agents on particular functions of individual cell types [14]. Most attention has been paid to pulmonary endothelial cells where a number of functions have been measured including 5-hydroxytryptamine uptake [4], angiotensin converting enzyme [15] and adenosine uptake [16]. While most work has concentrated on 5-hydroxytryptamine accumulation, the use of this marker as a measure

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of endothelial cell function is not clearly established (reviewed in Ref. 17). As recent results with adenosine accumulation appeared promising, we have, in the current study, used this as a marker of endothelial cell function. The integrity of the alveolar epithelial cells has been assessed by their ability to accumulate spermidine. Endogenous polyamines, such as putrescine, spermidine and spermine are accumulated by a specific transport process in alveolar epithelial cells [18] and this accumulation is reduced by agents, such as the herbicide, paraquat, which damage alveolar epithelial cells [19].

In this study we have shown that pretreatment with phorone causes an initial depletion followed by an elevation in both pulmonary and hepatic GSH. This elevation in GSH is associated with a marked protection against ANTU-induced toxicity to rat lung but does not protect against the toxicity of paraquat *in vivo*.

MATERIALS AND METHODS

Male Wistar rats (180–220 g) were obtained from Harlan-Olac Ltd (Southampton, U.K.). All animals were allowed food and water *ad lib*. ANTU was a generous gift of Dr Y. S. Bakhle (Royal College of Surgeons, London, U.K.). Paraquat dichloride was a gift from I.C.I. plc (Macclesfield, U.K.). Halothane was purchased from May and Baker (Dagenham, U.K.). Phorone was obtained from the Aldrich Chemical Co. (Gillingham, U.K.). Reduced glutathione (GSH) was obtained from Boehringer-Mannheim GmbH (Lewes, U.K.). [^{14}C]Spermidine trihydrochloride (1.17 μCi/mmol) and [2,^{3}H]adenosine (26 Ci/mmol) were purchased from Amersham International plc (Amersham, U.K.). All other chemicals were of the highest purity available and, unless otherwise specified, were obtained from the Sigma Chemical Co. (Poole, U.K.).

Treatment of animals. Phorone (200 mg/kg) was injected i.p. in corn oil (0.1 mL/100 g body weight). At the appropriate times, animals were killed by terminal anaesthesia using halothane. ANTU (5–20 mg/kg) was injected i.p. as a suspension in corn oil. Determination of hydrothorax and wet : dry lung weight ratios, and effects on adenosine and spermidine accumulation were carried out 4 hr after ANTU administration. The lethality of ANTU was assessed in control and phorone-pretreated rats. Animals were monitored six times daily for toxic signs. Hydrothorax was determined by quantitating the volume of fluid that could be aspirated from the thoracic cavity with a pasteur pipette. Lung wet : dry weight ratios were obtained by weighing after preparation of tissue and then heating (110°) to constant weight. Paraquat (20 mg paraquat ion/kg) was administered subcutaneously in 0.9% saline (w/v).

Measurement of lung non-protein sulphydryls (NPSH). Portions of lung (~100 mg) were homogenized at 4° using a Polytron homogenizer for 30 sec in 6.5% TCA/5 mM EDTA. NPSH was assayed using the fluorimetric method of Hissin and Hilf [20] using *o*-phthaldialdehyde. Fluorescence was detected with a Perkin-Elmer Fluorescence Spectrophotometer. NPSH levels are reported as GSH levels, since previous work has shown that lung contains only small amounts of acid-soluble thiols other than GSH [21].

Measurement of hepatic non-protein sulphydryls (NPSH). Livers were perfused *in situ* with ice-cold isotonic saline (0.9% w/v) and excised. Tissue was homogenized and NPSH determined using a similar method to that described for lung.

Perfusion of lungs and preparation of lung slices. Lungs were perfused *in situ* with Krebs–Ringer phosphate buffer, pH 7.4 (KRP) containing glucose (11 mM), using a single-pass perfusion system employing a Watson-Marlow 502S peristaltic pump. Perfusion was carried out in order to minimize erythrocyte contamination.

Adenosine and spermidine accumulation. Accumulation was assessed using lung slices. Immediately following perfusion, the largest lung lobe was excised and slices prepared using a McIlwain tissue chopper as described previously [22]. Lung slices (20–40 mg) were incubated with 0.1 μCi of radiolabelled spermidine and 10 μM unlabelled spermidine in 3 mL KRP for 30 min at 37° in a shaking water bath, as described previously [18]. Lung slices were washed, blotted and dissolved in 0.5 M KOH (400 μL) neutralized with 0.5 M HCl (400 μL) and radioactivity was determined, as described previously [23]. Adenosine uptake was determined in a similar manner. Radiolabelled adenosine (1 μCi) was incubated with 10 μM unlabelled adenosine for 15 min and tissue radioactivity was determined.

Statistical analysis. Student's *t*-test (unpaired) and analysis of variance (ANOVA) were used to evaluate significant differences between two groups and between treatments, respectively, at a significance level of $P \leq 0.05$.

RESULTS

Modulation of pulmonary and hepatic non-protein sulphydryl levels by phorone

Following administration of phorone (200 mg/kg, i.p.), both lung and liver showed similar changes in non-protein sulphydryl levels (Fig. 1). An initial depletion was followed by a marked increase in both organs and then a return to normal levels by 72 hr post-administration. The initial depletion and subsequent elevation in NPSH were maximal at approximately 4 and 48 hr post-administration, respectively. Both hepatic and pulmonary non-protein sulphydryl levels in control animals were similar to values reported in the literature [21].

Effect of phorone pretreatment on the toxicity of ANTU

In control animals, ANTU (10 mg/kg) was fatal to four out of eight (4/8) animals. This is consistent with previous investigations which have assigned an LD_{50} for ANTU at 7.5–10 mg/kg [6, 24]. The highest dose of ANTU (20 mg/kg) was fatal to all eight (8/8) animals. Both groups exhibited severe toxic signs including hunching and piloerection. The lowest dose of ANTU (5 mg/kg) was not fatal to any of eight animals (0/8) but toxic signs were still observed, though their severity was reduced

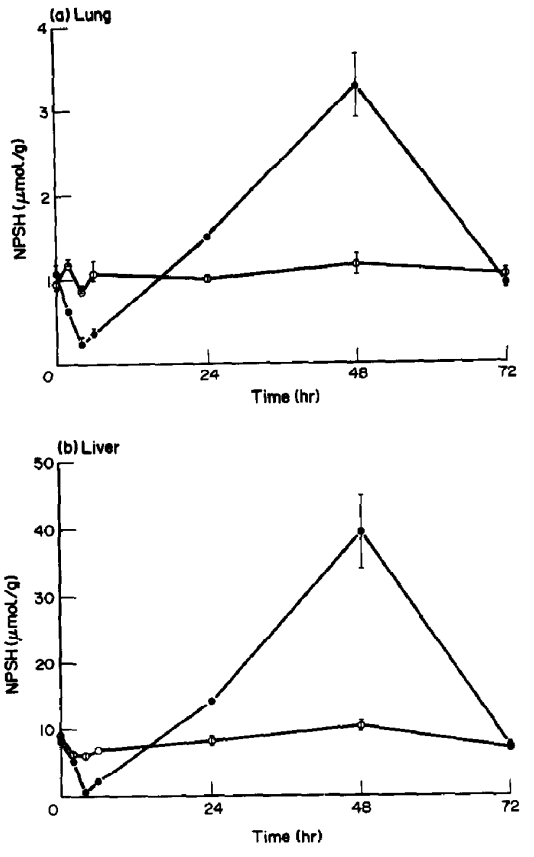

Fig. 1. Effects of phorone pretreatment on pulmonary and hepatic non-protein sulphydryl levels. Animals were injected i.p. with phorone (200 mg/kg in corn oil) and killed at the indicated times (●). Control animals were given corn oil alone (○). Lung and liver homogenates were prepared and non-protein sulphydryl levels assayed as described in Materials and Methods. Results represent the means ± SE of three experiments (N = 3).

compared with those of animals given the higher doses.

In animals given phorone 48 hr prior to ANTU, the ANTU-induced lethality was abolished completely (Table 1). This suggests that the elevation of pulmonary GSH levels obtained with phorone pretreatment protects against ANTU-induced lethality.

ANTU and hydrothorax

In control animals, ANTU produced a dose-dependent hydrothorax which was abolished entirely in rats pretreated with phorone (Table 1). The values for hydrothorax volume obtained were in good accord with those reported previously by Boyd and Neal [6].

After 4 hr, ANTU (10 mg/kg) induced a small increase in the wet:dry weight ratio of whole lung. This increase was reduced by prior treatment with phorone. No increase was observed for sliced lung wet:dry weight ratios following ANTU, suggesting that much of the oedematous fluid was released during the preparation of the slices (Table 1).

Effects of ANTU on adenosine accumulation

Rat lung slices accumulated radioactivity associated with adenosine in a time- and concentration-dependent manner (results not shown). Phorone pretreatment produced no significant modulation of the uptake of adenosine (10 μM) into lung tissue compared with that into lung slices prepared from control animals (Fig. 2). Pretreatment with ANTU resulted in a dose-dependent loss of ability to accumulate radiolabel, in control animals (Fig. 2). Pretreatment with phorone completely prevented the loss of adenosine uptake at the lower dose of ANTU (5 mg/kg) and almost completely prevented the loss at the higher dose of ANTU (10 mg/kg) (Fig. 2).

Spermidine accumulation

Spermidine was accumulated into lung slices prepared from control animals in a manner consistent with that seen in previous studies [18, 23]. Prior

Table 1. Influence of phorone pretreatment on ANTU toxicity in rat lung

Treatment	Mortality	Hydrothorax (mL)	Lung wet:dry weight ratios	
			Whole lung	Sliced lung
Control	0/8	ND	5.44	4.14
Phorone	0/8	ND	4.62	5.17
5 mg/kg ANTU	0/8	0.8–2.5	5.76	5.19
10 mg/kg ANTU	4/8	4.0–4.5	6.38	5.49
20 mg/kg ANTU	8/8	—	—	—
Phorone + 5 mg/kg ANTU	0/8	ND	5.20	5.10
Phorone + 10 mg/kg ANTU	0/8	ND	5.81	5.16
Phorone + 20 mg/kg ANTU	0/8	—	—	—

Mortality figures represent the number of deaths/total group size. Hydrothorax represents the volume of fluid aspirated and is the range of values obtained in three experiments. Lung wet:dry weight ratios are from one experiment typical of several.

ND, not detectable; — not determined.

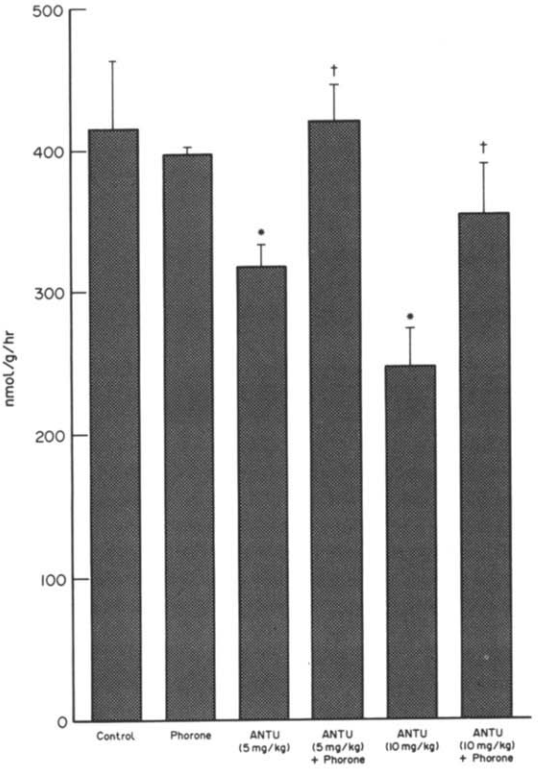

Fig. 2. Effect of ANTU on adenosine accumulation into lung slices from control and phorone treated rats. Animals were injected i.p. with corn oil alone or phorone (200 mg/kg) 48 hr prior to administration of ANTU (5 and 10 mg/kg, i.p.). At 4 hr after dosing with ANTU, lung slices were incubated with 1 μCi [2,-^{3}H]adenosine (10 μM). After a 15 min incubation period, adenosine-related radiolabel accumulated by the lung slice was determined as described in Materials and Methods. Results represent means ± SE (N = 3). * Denotes a significant difference ($P < 0.05$) between results from control animals (without ANTU) and those given ANTU, without prior treatment with phorone. † Denotes a significant difference ($P < 0.05$) between rats given phorone + ANTU and those given ANTU alone.

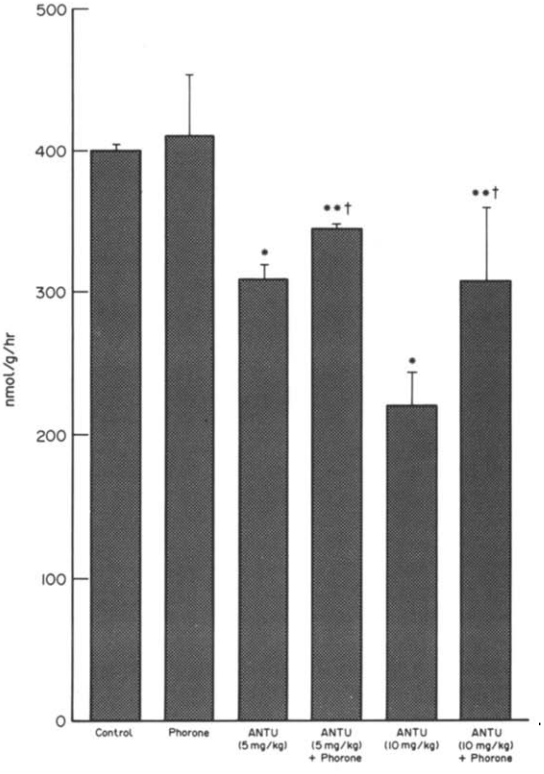

Fig. 3. Effect of ANTU of spermidine accumulation in lung slices from control and phorone-pretreated rats. Animals were treated as described in the legend to Fig. 2. Lung slices were incubated with 0.1 μCi [^{14}C]spermidine (10 μM) for 30 min and its uptake determined as described in Materials and Methods. Results represent means ± SE (N = 3). * Denotes significant difference ($P < 0.05$) between control animals (without ANTU) and those given ANTU without prior treatment with phorone. ** Denotes significant difference ($P < 0.05$) between rats give phorone alone and those given phorone + ANTU. † Denotes significant difference ($P < 0.05$) between rats given phorone + ANTU and those given ANTU alone.

treatment with phorone did not affect the uptake of the oligoamine. In the absence of phorone pretreatment, ANTU (5 and 10 mg/kg) resulted in a significant dose-dependent reduction of spermidine uptake (Fig. 3). Pretreatment with phorone protected partially but not completely against the loss of spermidine uptake caused by ANTU (Fig. 3).

Effects of phorone pretreatment on the toxicity of paraquat

Paraquat (20 mg/kg) resulted in two fatalities out of six in control animals (2/6). Pretreatment with phorone (200 mg/kg in corn oil, i.p.) did not protect against paraquat toxicity, if anything, a slight increase in toxicity was observed [three fatalities out of six animals (3/6)]. Treatment with phorone alone (200 mg/kg) was not fatal to any of six animals.

DISCUSSION

The most significant finding in the present study was that prior treatment with phorone conferred marked protection against the toxicity of ANTU. Protection was observed against ANTU-induced lethality, hydrothorax and changes in adenosine and spermidine uptake.

Whilst the protection against ANTU-induced toxicity was clear, the precise mechanism for this protection is less clear. Lee *et al.* [7] proposed that ANTU is activated metabolically to atomic sulphur, a portion of which becomes covalently bound to the cysteine side chain of proteins forming the hydrodisulphide. Therefore, GSH may protect by offering an alternative site for covalent binding of the reactive metabolite(s) of ANTU. In our studies, we used pretreatment with phorone to increase pulmonary GSH (Fig. 1) in order to determine if this could protect the animals against ANTU-induced toxicity. Phorone rather than diethyl maleate was chosen because it has been reported to exert fewer effects on cytochrome P450 monooxygenase activity [25]. The intracellular pulmonary distribution of the

elevated GSH following phorone pretreatment is unknown. The lung is a heterogeneous organ comprising over 40 different cell types [26]. Recent studies in our laboratory have suggested that much of the GSH is in cell types other than the alveolar epithelium, possibly including the endothelium [27]. In addition, in isolated cell preparations from rabbit lung the rate of *de novo* GSH synthesis was lower in type II cells than Clara cells and alveolar macrophages [28]. Thus, the elevation of GSH following phorone pretreatment may have occurred, in part, in endothelial cells, resulting in the observed protection against ANTU. This was also supported by the observations that phorone pretreatment did not protect against paraquat toxicity *in vivo* and paraquat damages primarily alveolar epithelial type I and type II cells [29].

A further complication in delineating the mechanism of the phorone protection against ANTU-induced toxicity was the striking elevation in hepatic NPSH levels (Fig. 1). These changes could alter the pharmacokinetics of ANTU resulting in a decreased availability to the lung and therefore a decreased toxicity. Recent work has also shown that phorone may change heme oxygenase activity in rat liver [30], possibly altering hepatic metabolism of ANTU and, thus, altering its bioavailability to the lung. In addition, both lung and liver microsomes may activate ANTU [7] and, therefore, a possible hepatic contribution to the observed protection cannot be excluded.

Despite the problems involved in understanding the precise mechanism of the protection afforded by phorone pretreatment against ANTU-induced toxicity, similar pretreatment may also protect against other types of endothelial cell damage such as that induced by hyperoxia.

In the current studies we employed the accumulation of adenosine as a functional marker of endothelium integrity (see also introduction). In our laboratory, under the conditions stated here, the loss of ability to accumulate adenosine correlates well with endothelial damage (unpublished results). In this investigation, adenosine uptake was clearly and significantly diminished by ANTU in a dose-dependent manner, and this loss of uptake was ameliorated almost entirely in lung slices prepared from phorone-pretreated rats (Fig. 2). It should be noted that, although we refer to adenosine accumulation, under the conditions used in our experiments adenosine was metabolized rapidly to a number of products including ATP (unpublished results), in agreement with studies using isolated perfused lung [16]. Thus, it would be more accurate to refer to radioactivity associated with adenosine and its metabolic products rather than adenosine accumulation.

ANTU (5 and 10 mg/kg) also caused a dose-dependent inhibition of spermidine accumulation (Fig. 3). As spermidine accumulation occurs in alveolar epithelial as opposed to endothelial cells, these results suggest that ANTU may also damage epithelial cells. This agrees with the results of Meyrick *et al.* [3] although they observed these histological effects at ANTU doses of 50 mg/kg. In the present study, ANTU did not show a particular selectivity for the endothelium compared with the epithelium, based on functional markers. This could be the result of assessing these parameters 4 hr after ANTU administration (when oedema is maximal [6]). Investigation at earlier times might have shown preferential damage to particular cell types.

The failure to protect rats against the toxicity of paraquat *in vivo* is intriguing, since recent studies [27, 31, 32] have implicated GSH in its mechanism of toxicity. The rate of *de novo* GSH synthesis is low in rabbit alveolar epithelial type II cells [28]. Phorone presumably elevates GSH by rebound synthesis which may be low or absent in type II cells.

In summary, we have shown that phorone pretreatment results in a marked elevation of thiol levels in both lung and liver. Prior treatment with phorone did not protect rats against paraquat but protected markedly against the lethality and pulmonary toxicity of the endothelial damaging agent ANTU.

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