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Potentiation by piperonyl butoxide of alpha-naphthylthiourea toxicity in the isolated, perfused rat lung

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The rodenticide alpha-naphthylthiourea (ANTU) produces pulmonary toxicity in many animal species [1–3]. This toxicity is characterized by capillary endothelial cell damage, pulmonary edema and pleural effusion [4, 5]. Although the precise mechanism by which ANTU injures lungs is unknown, evidence exists which suggests that toxicity may result from the action of a metabolite of ANTU and not from ANTU itself. *In vitro*, binding of [^{35}S]ANTU and [^{14}C]ANTU to rat lung and liver microsomes is stimulated in the presence of an NADPH-generating system [6, 7]. *In vivo*, pretreatment of rats with doses of ANTU which inhibit lung mixed-function oxidase activity decreases both the toxicity and the covalent binding of subsequently administered [^{35}S]ANTU to lung and liver macromolecules [6]. Resistance to ANTU toxicity is also produced by pretreatment of rats with the cytochrome P-450 inhibitor piperonyl butoxide [8]. These data suggest that a cytochrome P-450 monooxygenase-catalyzed metabolite(s) of ANTU is responsible for toxicity. The purpose of this study was to determine if perfusion with ANTU produces injury to isolated rat lungs and whether piperonyl butoxide prevents ANTU pneumotoxicity in this preparation as it has been reported to do *in vivo*.

Materials and methods

Lungs were isolated from Sprague–Dawley male rats (Charles River Laboratories, Portage, MI) weighing 250–350 *g*, as previously described [9]. They were anesthetized with 50 mg/kg sodium pentobarbital (i.p.) and were given 500 units heparin in the inferior vena cava before the trachea and pulmonary artery (PA) were cannulated. After the heart was cut away, the lungs were removed from the thoracic cavity and suspended by the PA cannula in a 37° chamber. They were ventilated at a rate of 80–90 cycles/min with a warmed, humidified gas mixture of 95% O_2/5% CO_2 using a small animal respirator (Mallard Medical Co., Irvine, CA). Inspiratory pressure was 13–16 cm H_2O, and positive end expiratory pressure was maintained at 2–3 cm H_2O. Krebs–Ringer bicarbonate buffer (KRBSA) containing 4% bovine serum albumin (Miles Biochemicals Inc., Elkhart, IN) was pumped into the PA at 10 ml/min with a Masterflex pump (Cole Parmer Instrument Co., Chicago, IL). PA pressure was monitored with a Statham P23ID pressure transducer (Statham Instruments, Hato Rey, PR) and recorded by a Grass model 7B Polygraph (Grass Instrument Co., Quincy, MA).

Lungs were initially perfused in a single-pass manner for 10 min to clear blood from the vasculature and to stabilize perfusion pressure. After this time, lungs were perfused in a recirculating manner with KRBSA containing 1-(1-naphthyl)-2-thiourea (ANTU, 50 μM; Pfaltz & Bauer, Stamford, CT) or its vehicle (*N*,*N*-dimethylformamide, DMF; Sigma Chemical Co., St. Louis, MO). In some experiments, piperonyl butoxide (1 mM; ICN Biomedicals, Inc., Plainview, NY) or saline (0.9%) was added to the recirculating KRBSA 10 min prior to the addition of ANTU or DMF vehicle to the medium. Perfusions were carried out for 90 min after the addition of ANTU or DMF. After 90 min, lungs were perfused in a single-pass manner for an additional 10 min with KRBSA containing 0.1 μM 5-hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate ([^{14}C]5-HT; 58 mCi/mmol; Amersham, Arlington Heights, IL). Samples of effluent medium were collected 5 and 10 min after switching to the [^{14}C]5-HT-containing medium. The samples were passed through Biorex 70 (pH 6.0; Bio-Rad Laboratories, Richmond, CA) columns to separate 5-HT from its acid metabolites as previously described [9]. Radioactivity in samples was measured by a Beckman model CS-3150P scintillation counter. The percentages of 5-HT removed and metabolized in a single pass through the lungs were calculated as previously described [10]. After 10 min of perfusion with 5-HT, perfusions were terminated, lungs were weighed, and the relative lung weight (LW/BW) was determined.

In experiments with two groups, means were compared by Student's *t*-test [11]. A completely random analysis of variance was used to analyze data from experiments involving four groups. Means were compared using the least significant difference test. When variances were not homogeneous, log transformations were performed to render them so. Data are presented as the mean ± SEM. The criterion for significance was $P < 0.05$.

Results and discussion

The effects of ANTU on the isolated, buffer-perfused rat lung (IPL) are shown in Table 1. The relative weight of lungs perfused with ANTU (50 μM) was significantly greater than that of lungs perfused with DMF vehicle. Perfusion pressure also increased in lungs perfused with ANTU. Although the percentage of 5-HT which was metabolized after both 5 (Table 1) and 10 min (data not shown) of perfusion with this amine was decreased by preperfusion with ANTU, the fraction of 5-HT removed in a single pass through the pulmonary vasculature was unaltered at either time. Although the mechanism of this effect is not known, one possibility is that ANTU may inhibit a monoamine oxidase that metabolizes 5-HT.

In contrast to our results, Block and Schoen [12] demonstrated that lungs from rats that had been treated with ANTU *in vivo* 4 hr prior to isolation removed less 5-HT than controls. Differences in the results obtained in the two

Table 1. Effect of ANTU on the isolated, perfused rat lung

	Treatment	
Index of injury	DMF	ANTU
LW/BW × 10^3	4.51 ± 0.20	5.70 ± 0.36*
Δ P	−0.4 ± 0.1	1.6 ± 0.3*
% 5-HT removed	79.2 ± 0.6	75.9 ± 2.1
% 5-HT metabolized	41.3 ± 0.8	35.1 ± 1.2*

Lungs were perfused with ANTU (50 μM) or an equal volume of DMF vehicle as described in Materials and Methods. Relative lung weight (LW/BW × 10^3) was determined after 100 total min of perfusion. Δ P = pressure at the end of perfusion minus pressure before the addition of DMF or ANTU to the perfusion medium. Data reported for % 5-HT removed and metabolized were obtained from samples of effluent medium collected after 5 min of perfusion with 5-HT. Initial pressures and body weights of rats were not different between the two groups. Values are means ± SEM; N = 7 for each group.

* Significantly different from DMF control.

Table 2. Effect of piperonyl butoxide on lung injury induced by ANTU

Treatment	LW/BW × 10^3	Δ P
Saline/DMF	4.62 ± 0.11	−0.6 ± 0.2
Piperonyl butoxide/DMF	5.48 ± 0.32	−0.9 ± 0.3
Saline/ANTU	5.82 ± 0.39*	1.0 ± 0.3*,†
Piperonyl butoxide/ANTU	10.26 ± 0.98*,†,‡	0.7 ± 0.4*,†

Lungs were perfused with ANTU (50 μM) or an equal volume of DMF and piperonyl butoxide (1 mM) or an equal volume of saline as described in Materials and Methods. Relative lung weight (LW/BW × 10^3) was determined after 100 total min of perfusion. Δ P = pressure at the end of perfusion minus pressure before the addition of ANTU or DMF to the perfusion medium. Initial pressures and body weights of rats were not different between groups. Values are means ± SEM. N = 6 for each group not perfused with ANTU. N = 8 for each group perfused with ANTU.

* Significantly different from lungs perfused with saline and DMF.
† Significantly different from lungs perfused with piperonyl butoxide and DMF.
‡ Significantly different from lungs perfused with saline and ANTU.

studies may be a consequence of the manner in which ANTU was administered. In addition, factors not present in the IPL preparation may influence the toxicity of ANTU when it is administered *in vivo*.

When pretreated *in vivo* with chemicals that inhibit cytochrome P-450 monooxygenases (i.e. piperonyl butoxide or small, nonlethal doses of ANTU), rats are protected against ANTU pneumotoxicity [6, 8]. These data suggest that the toxicity of ANTU *in vivo* is mediated by a metabolite formed by a cytochrome P-450 monooxygenase. To investigate whether toxicity to the IPL was mediated by ANTU or a metabolite(s) formed via the action of pulmonary cytochrome P-450, we perfused lungs with ANTU and piperonyl butoxide (PBT, 1 mM). Cytochrome P-450-mediated metabolism of several compounds in the rat is inhibited by this concentration of PBT. The IC_{50} of PBT for ethylmorphine, nitroanisole or aniline metabolism by rat liver microsomes is 40 μM or less [13]. At 1 mM, metabolism of ethylmorphine by rat liver microsomes is inhibited by more than 75% [14]. In addition, cytochrome P-450-dependent covalent binding of 4-ipomeanol to microsomes from rat lungs is inhibited completely by 1 mM PBT [15].

In contrast to results obtained *in vivo* by Van Den Brink and coworkers [8], PBT afforded no protection against ANTU toxicity in the isolated lung (Table 2). In fact, the relative weight of lungs co-perfused with PBT and ANTU was significantly greater than that of lungs co-perfused with ANTU and the vehicle for PBT (saline). Thus, PBT seemed to potentiate the toxicity of ANTU in the IPL. Although the change in perfusion pressure of lungs co-perfused with PBT and ANTU was greater than that of controls, it was not greater than that of lungs co-perfused with ANTU and saline. These data suggest that the additional fluid accumulation in lungs perfused with ANTU and PBT was not induced by an increase in perfusion pressure. The ability of lungs perfused with PBT and ANTU to take up or to metabolize 5-HT was not different from that of lungs perfused with ANTU and saline (data not shown). Thus, PBT did not influence the ability of ANTU to compromise this metabolic function of pulmonary vascular endothelial cells.

Why piperonyl butoxide potentiates ANTU toxicity in the isolated lung preparation but attenuates toxicity *in vivo* [8] is unknown. One possible explanation for the disparate results is that metabolites of ANTU which are formed by the liver may be pneumotoxic and those formed by the lung may be nontoxic. If so, then PBT given *in vivo* may reduce the formation of toxic, hepatic metabolites of ANTU that injure the lung. Another possibility is that metabolism of ANTU is not required for it to produce pneumotoxicity, and PBT influences toxicity by some other mechanism. Additional studies are needed to test these possibilities.

In conclusion, ANTU can produce toxicity to rat lungs, and this does not require extrapulmonary metabolism. In addition, our results suggest that ANTU pneumotoxicity does not require bioactivation by isozymes of pulmonary cytochrome P-450 which are sensitive to piperonyl butoxide.

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