Premature Rats Treated with Propylthiouracil Show Enhanced Pulmonary Antioxidant Enzyme Gene Expression and Improved Survival during Prolonged Exposure to Hyperoxia

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ABSTRACT

In full-term newborn rats, propylthiouracil (PTU) treatment has been previously shown to decrease susceptibility to O_2-induced lung damage and improve survival during hyperoxic exposure. However, no differences were found in lung antioxidant enzyme (AOE) activity responses to hyperoxia compared with O_2-exposed untreated (control) term rats. To further explore possible pulmonary protective effects of PTU treatment in prematurely delivered animals, we administered PTU (0.015%) in drinking water to timed-pregnant rats for the final 10 d of gestation prior to delivery 1 d before term, and during lactation; control pregnant/nursing rats received untreated water. Both groups of 21-d premature rat pups were randomized to either >95% O_2 or room air exposure after birth for up to 14 d. The left lungs of 7-d exposure pups were used to quantitate the concentrations of AOE mRNA by solution hybridization; the right lungs of the same pups were assayed for AOE activities. PTU treatment resulted in survival rates of O_2-exposed preterm rat pups that were consistently higher at all time periods in hyperoxia including 7 d [PTU, 67 of 82 (82%) *versus* control pups, 58 of 113 (51%); $p < 0.001$] and 14 d [PTU, 31 of 39 (79%) *versus* control, 15 of 66 (23%); $p < 0.001$]. Further evidence of increased tolerance to >95% O_2 in PTU pups included a significant decrease in the incidence of microscopic intraalveolar edema and a significant increase in lung tissue surfactant-related phospho-lipids compared with O_2-exposed control pups. At 7 d in high O_2, the PTU-treated pups showed greater increases in the lung AOE mRNA levels and AOE activities of catalase and glutathione peroxidase in response to hyperoxia compared with the untreated control O_2 group. Thus, we conclude that PTU treatment protects premature rats against O_2-induced lung injury and lethality during prolonged hyperoxic challenge. The protective action of PTU may be related, at least in part, to the enhanced pulmonary AOE gene expression in the treated rat pups with resultant increases in protective AOE activity levels in response to neonatal lung oxidant challenge. (*Pediatr Res* **38: 292–297, 1995**)

Abbreviations
PTU, propylthiouracil
AOE, antioxidant enzyme(s)
SOD, superoxide dismutase
CuZnSOD, copper-zinc superoxide dismutase
MnSOD, manganese superoxide dismutase
CAT, catalase
GP, glutathione peroxidase
DSPC, disaturated phosphatidylcholine
TRH, thyrotropin-releasing hormone
T₃, triiodothyronine
T₄, thyroxine

PTU is a thioamide derivative that inhibits thyroid hormone synthesis and readily crosses the placenta (1). When PTU was administered to pregnant rats, their late gestation fetuses had significantly increased pulmonary AOE (SOD, CAT, and GP) activities compared with age-matched control fetuses, whereas the lung surfactant contents were similar (2). Adult rats pretreated with PTU showed less O_2-induced lung damage and

lethality during exposure to hyperoxia than control rats, as well as greater pulmonary reduced glutathione levels and CAT activity in response to high O_2 exposure (3). In a full-term newborn rat study, PTU treatment was recently shown to decrease susceptibility to O_2-induced lung damage and consistently improve survival during neonatal hyperoxic exposure; however, no differences were found in the lung AOE activity responses to hyperoxia in the term O_2-exposed PTU-treated pups compared with O_2-exposed control term rats (4).

To specifically explore possible pulmonary protective effects of PTU in preterm animals, we used a prematurely delivered rat model (previously demonstrated to have a transiently improved hyperoxic survival *versus* the term rat as well as a

greater magnitude of hyperoxic induction of lung AOE activities) (5) and examined comparative hyperoxic survival, parameters of pulmonary O_2 toxicity, and lung AOE responses to hyperoxia in the growing lungs of PTU-treated and control preterm animals. On the basis of the previous thyroid hormone and anti-thyroid drug treatment studies, we hypothesized that premature rats treated with PTU would demonstrate superior tolerance to prolonged hyperoxic exposure and an associated decreased evidence of O_2-induced lung injury.

METHODS

Animals and treatment. Adult female and male breeder Sprague-Dawley albino rats were obtained from Charles River Laboratories (Wellington, MA). Breeding was accomplished by placing two female rats in the same cage with one male overnight. Probable pregnancy was determined by a positive vaginal sperm smear the next morning, and the onset of gestation was considered to be the midpoint of the cohabitation period. The timed-pregnancy rats were maintained on standard laboratory food and water *ad libitum* and were kept on a 12-h light/dark cycle in the accredited University of Miami Animal Care Facilities. All portions of the experimental protocol were preapproved by the University of Miami Animal Research Welfare Committee.

Ten days before preterm delivery at gestational d 21 (term = d 22 of gestation), timed-pregnancy rats were randomly assigned to either a PTU treatment group or a control group. PTU (Sigma Chemical Co., St Louis, MO), 0.015%, was administered in the drinking water, which was made up fresh daily. The control group received drinking water not containing PTU. This regimen has been used previously by others and has been shown to be effective in inhibiting thyroid hormone synthesis without influencing litter size or viability (6). The procedures and dosage of PTU were identical to those used in previously reported full-term newborn rat studies from our laboratories [calculated maternal dose of PTU $\approx$ 30 mg/kg/d (approximately twice the orally administered clinical dosage)]. PTU treatment of mother rats during the final 10 d of gestation and throughout lactation resulted in inhibited serum T_3 and T_4 levels in their full-term offspring (4).

At 21 d of gestation, pregnant rats were anesthetized by intraperitoneal injection of ketamine/xylazine (ketamine:xylazine, 45 mg/kg:5 mg/kg) and hysterotomy was performed. The newly delivered pups were rapidly resuscitated by removing the surrounding membranes, drying them vigorously, warming them on heating pads, and applying low flow O_2 (~30%) for ~30 min.

Hyperoxic exposure. Shortly after delivery and stabilization, all the surviving 21-d premature pups of 3–5 equivalently treated litters were first pooled together and then randomly redistributed to surrogate equivalently treated mother rats (which had recently delivered full-term PTU or control offspring). These equivalently treated surrogate mother rats were then continued on the same treatment during lactation. For each group (PTU-treated or untreated control), one half of the reconstituted litters was placed in >95% O_2 exposure chambers and one half remained in room air. Some non-

experimental full-term newborn rat pups (marked by tail snip) were added at 24 h and/or 48 h of exposure when necessary to readjust the litter sizes to 12 pups per dam to ensure equal litter sizes for equivalent nutrition. The procedures and conditions of hyperoxic exposures were as previously described (5). Survival was noted daily during the 14-d exposure period.

Biochemical analyses. Randomly selected O_2-exposed and air control pups from each group were killed by an intraperitoneal overdose of pentobarbital after 7 d of exposure. The procedures of lung perfusion were as previously described (5). The left lungs were used for measuring AOE mRNA concentrations, and the right lungs of the same pups were used for assaying AOE activities.

AOE mRNA quantitation. The rat complementary RNA for CuZnSOD, MnSOD, CAT, and GP were prepared as previously described (7–9). Total nucleic acids were extracted by phenol-chloroform extraction of the left lung tissue homogenate (9). Quantitation of each AOE mRNA was achieved using the solution hybridization assay of Durnam and Palmiter (10). Results of the AOE mRNA concentrations were expressed as the specific AOE mRNA molecules per mg DNA.

AOE activity assays. The right lungs were homogenized in cold 2.5 mM potassium phosphate buffer (pH 7.8) in an Omni homogenizer (Omni International Inc., Waterbury, CT). The homogenates were centrifuged at 27 000 $\times$ *g* for 45 min at 4°C. The supernatant fractions were then dialyzed overnight against three changes of 50 vol of 2.5 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA and were subsequently used for analyses of AOE activities using standard spectrophotometric assays for SOD (cytochrome *c* reduction rate by xanthine-xanthine oxidase) (11), CAT (H_2O_2 consumption rate) (12), and GP (NADPH oxidation rate with cumene hydroperoxide substrate) (13). The method of differential cyanide inhibition was used to individually quantitate cytosolic CuZnSOD and mitochondrial MnSOD activities (14). All enzyme activities were expressed as activity units per mg DNA. DNA in aliquots of lung homogenates used for AOE mRNA or for AOE activity was extracted and measured using purified calf thymus DNA (Sigma Chemical Co.) as a standard (15).

Surfactant phospholipids and DSPC. The lipids were extracted from lung homogenates using the method of Bligh and Dyer (16). A portion of the lipid extracts was used to isolate DSPC according to Mason *et al.* (17). The total phospholipids and DSPC content were measured by phosphorus determinations in these fraction (18). A known quantity of [^{14}C]dipalmitoyl-phosphatidylcholine (NEN, Boston, MA) was added before lipid extraction to estimate and correct for sequential losses. Lipids were expressed as milligrams/mg of protein. Protein was measured by the Folin-phenol reagent method (19).

Lung pathologic analyses. Randomly selected animals from each group at 14 d of exposure were killed as above. Unperfused, uninflated left lungs were tied off and removed for assessing lung wet weight/dry weight ratios [80°C oven; lung dried until constant weight found (72 h)]. For microscopic studies, the right lungs of those animals were fixed *in situ* via a tracheal cannula at a constant inflation pressure of 20 cm H_2O (fixative, 10% buffered formalin). The hematoxylin and eosin-

stained lung sections (right upper, middle, and lower lobes) were first coded and then examined independently by two investigators in a blinded fashion for pathologic changes, looking for evidence of interstitial, perivascular/peribronchiolar, and intraalveolar edema, and lung hemorrhage.

Statistical analyses. Comparative survival data and incidence of intraalveolar edema were compared by χ^2 testing. Four group comparisons (PTU air, PTU O_2, control air, and control O_2) were made by analysis of variance and Duncan's multiple range test. Kramer's extension of Duncan's test was used in cases of unequal number of replications (20). For all statistical tests, a $p < 0.05$ value was considered a significant difference between the compared values.

RESULTS

Survival in hyperoxia. Initial survival at 30 min after premature delivery averaged 90% for both groups (170/189 for PTU pups and 223/247 for control pups, number alive/total number delivered). The composite survival rates of PTU-treated and control premature rat pups during 14 d of hyperoxia are illustrated in Figure 1. The survival rate of the PTU-treated pups when placed in >95% O_2 at birth was consistently higher at all time periods in hyperoxia from 1 to 14 d. However, extensive past experimental experience leads us to believe that the early deaths in hyperoxia (before at least 2 d of hyperoxia for preterm rat pups) are not due to pulmonary O_2 toxicity *per se*, because approximately 1/3 of the prematurely delivered rat pups fail to survive the first 48 h of life in room air as well (see legend to Fig. 1).

Lung biochemical responses to hyperoxia. The comparative responses of the pulmonary AOE to 7 d of hyperoxic challenge in the PTU-treated and control premature rat pups are shown in Figure 2 (for AOE activities) and Figure 3 (for AOE mRNA concentrations). After 7 d in hyperoxia, both PTU-treated and untreated controls were able to induce an adaptive lung AOE

activity response to hyperoxic exposure (except MnSOD). PTU pups exposed to hyperoxia demonstrated significantly greater increases in CAT and GP activities than the untreated control O_2 group. The results of lung AOE mRNA concentrations (using the contralateral lungs of the same group of animals) showed the four AOE mRNA levels significantly elevated in the PTU-treated O_2-exposed group compared with PTU-treated air-exposed pups and greater increases in the levels of MnSOD mRNA and GP mRNA in the PTU O_2 group compared with the untreated O_2 group.

The lung tissue DSPC and total phospholipid content responses to 7 d of hyperoxic exposure are summarized in Table 1. Offspring of PTU-treated dams had significant increases in lung tissue DSPC and total phospholipid contents compared with control offspring in prolonged hyperoxia.

Lung pathologic analyses. The comparative lung wet weight/dry weight ratios (reflection of pulmonary edema) after 14 d of hyperoxia showed no differences between the PTU-O_2 (5.95 ± 0.09, ↑ 17%) and control-O_2 (6.03 ± 0.12, ↑ 22%) groups (their respective air control group values were 5.11 ± 0.03 and 4.96 ± 0.02, mean ± SEM, n = 15–20 samples/group). However, when comparing pathologic evidence of more advanced O_2 toxicity, intraalveolar edema was found to be present in 46% (11 of 24) of the control group lung sections after 14 d of hyperoxia *versus* only 17% (4 of 24) of the PTU group lung sections ($p < 0.05$). Although the similar increases in wet/dry weights in the O_2 groups suggest endothelial cell injury was present in both groups, the intraalveolar edema results would seem to suggest a greater degree of injury to the alveolar epithelial cell barrier of lungs of the O_2-control group.

DISCUSSION

Prenatal administration of either thyroid hormone (T_3) or TRH to pregnant rats in late gestation produces significantly elevated lung DSPC concentrations, but significantly depressed
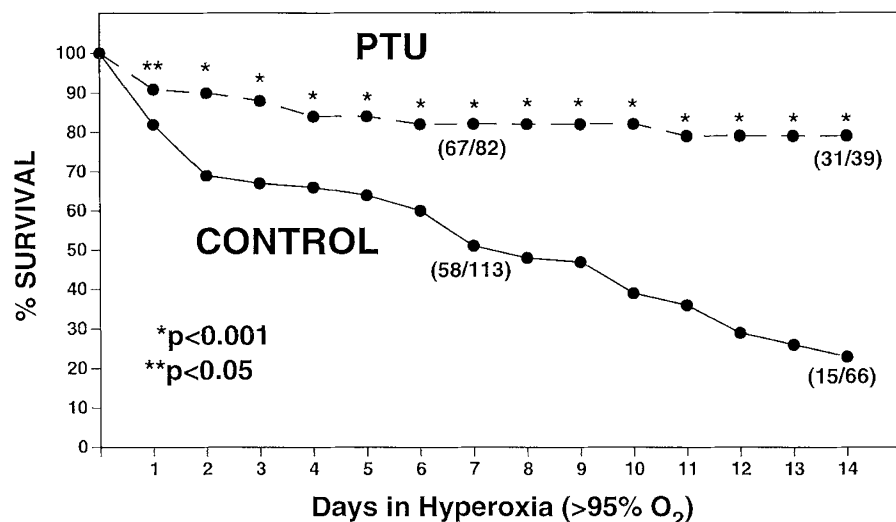

Figure 1. Survival of PTU-treated and control premature rat pups in >95% O_2 for 14 d (n/n, number alive/number put in O_2). *, $p < 0.001$ and **, $p < 0.05$ for the two O_2-exposed group survival rate comparisons. For the pups maintained in room air, the subsequent survival rates for the PTU-treated group were 81% (at 24 h), 75% (at 48 h), and 75% (2 to 14 d of exposure). For the untreated air control pups, the rates were 76% (at 24 h), 69% (at 48 h), and 69% (2–14 d of exposure).
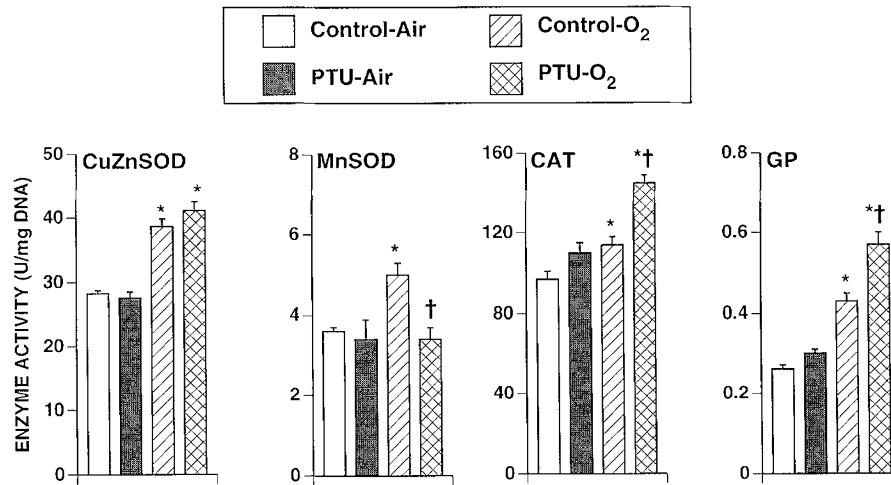
Figure 2. Comparative pulmonary AOE activity levels of PTU-treated and control premature rat pups at 7 d of hyperoxic (>95% O$_2$) or room air (21% O$_2$) exposure. Rat right lungs were assayed for CuZnSOD, MnSOD, CAT, and GP activities. Values are based on 6 lung samples/group; *bars* indicate mean ± SEM. *, $p < 0.01$ for O$_2$ group *vs* respective air group. †, $p < 0.01$ for PTU-O$_2$ group *vs* control-O$_2$ group values.
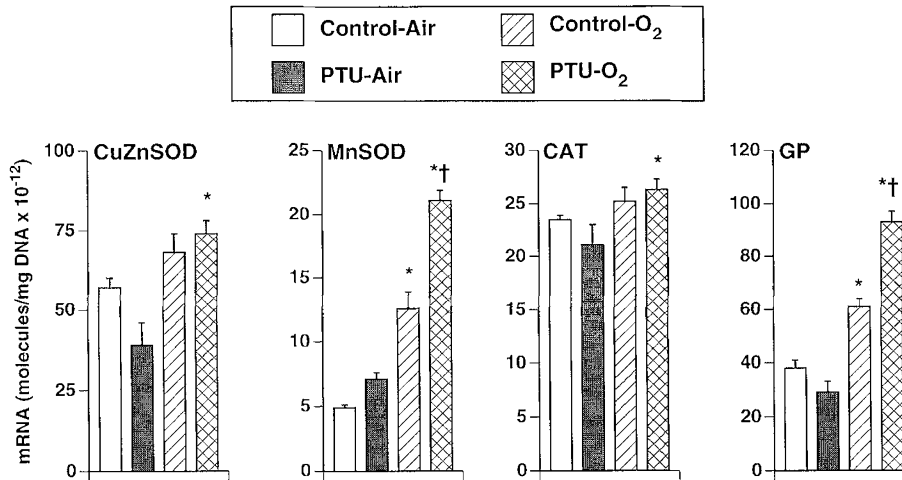

Figure 3. Comparative lung AOE mRNA concentrations of PTU-treated and control premature rat pups at 7 d of hyperoxic (>95% O$_2$) or room air (21% O$_2$) exposure. Rat left lungs from same samples as Figure 2 were assayed for CuZnSOD mRNA, MnSOD mRNA, CAT mRNA, and GP mRNA by solution hybridization using respective ^{35}S-labeled complementary RNA probes. Values are based on 6 lung samples/ group; *bars* indicate mean ± SEM. *, $p < 0.05$ for O$_2$ group *vs* respective air group. †, $p < 0.01$ for PTU-O$_2$ group *vs* control-O$_2$ group values.

Table 1. *Comparative lung surfactant content responses to 7 d of hyperoxic exposure*

	DSPC (mg/mg protein)	TPL (mg/mg protein)
Control-air	0.062 ± 0.002	0.312 ± 0.010
Control-O$_2$	0.054 ± 0.003 (↓13%)	0.286 ± 0.020 (↓8%)
PTU-air	0.068 ± 0.004	0.343 ± 0.008
PTU-O$_2$	0.080 ± 0.004*† (↑18%)	0.378 ± 0.008† (↑10%)

Values are mean ± SEM for four to five samples/group. TPL, total phospholipid.

* $p < 0.05$ *vs* PTU-air group.

† $p < 0.01$ *vs* control-O$_2$ group.

fetal lung AOE activities (21, 22). After prenatal TRH treatment newly delivered term rats are able, however, to increase their AOE activities markedly in response to hyperoxia, despite their lower baseline (at birth) AOE activity levels (22). Unex-

pectedly though, full-term TRH offspring were found to have comparatively poorer hyperoxic survival with increased indices of O$_2$-induced lung damage compared with control newborns in hyperoxia (23). In contrast, when endogenous thyroid hormone production was inhibited by maternal PTU administration, newborn term rats demonstrated decreased susceptibility to O$_2$-induced lung damage and improved survival during hyperoxic exposure not accompanied by increased lung AOE responses to hyperoxia compared with O$_2$-control term rats, nor by any decreased lung tissue O$_2$ radical formation in high O$_2$ (4).

We hypothesized in the present study that, similar to term rats, preterm rats treated pre- and postnatally with PTU would likewise demonstrate superior tolerance to prolonged hyperoxic exposure and an associated decreased evidence of O$_2$-induced lung injury. Indeed, in addition to the consistent and dramatically superior survival rate of the PTU-treated preterm offspring in hyperoxia, we found concomitant evidence of

increased protection against O_2-induced lung damage in the DSPC/phospholipid data and in the comparative intraalveolar edema results during O_2 exposure. These findings in premature rats are therefore in agreement with previous studies either in adult rats (3) or in full-term newborn rats (4), in which decreased susceptibility to O_2-induced lung damage and improved hyperoxic survival after PTU treatment were reported. However, our PTU-treated premature pups showed greater increases in their lung CAT and GP activities in response to hyperoxic exposure compared with the untreated control O_2 group, a finding consistent with results in adult rats (3). Our PTU-treated preterm animal results differ importantly from the findings in PTU-treated full-term newborn rats which respond to neonatal hyperoxic challenge with no greater elevations in pulmonary AOE activities than control-O_2 pups (4).

The discrepancies in the biologic/biochemical responses to hyperoxic exposure between full-term newborn and preterm rat pups are actually not as unique as they first appear to be. For example, whereas prenatal dexamethasone administration to preterm rats has been shown to enhance survival and increase the activities of lung CAT and SOD at birth, the same prenatal dexamethasone treatment to term rats did not produce changes in AOE activities (23). Similarly, prenatal TRH-treated full-term rat pups demonstrated decreased survival rates during hyperoxic exposure *versus* O_2-exposed controls (24); however, the same prenatal TRH treatment to preterm pups resulted in no significant differences in hyperoxic survival *versus* untreated control-O_2 premature animals (our unpublished observation). For the PTU studies, the differences in the premature *versus* term newborns' lung AOE responses to high O_2 exposure may suggest that the mechanism of PTU's protective effect against O_2 toxicity is related to the induction of pulmonary AOE in the preterm (*versus* term) animal model.

It is believed that thyroid hormone is able to bind to DNA sequences near to specific genes and thereby "regulate" (promote or inhibit) transcription of these genes—much like glucocorticoid hormone does (25, 26). The molecular biology studies herein were undertaken to provide insight into the mechanism by which PTU treatment might be affecting or altering gene expression of the protective AOE under hyperoxic conditions. Analyzing the data in Figures 2 and 3, we found that in untreated control preterm lungs primarily a pretranslational mechanism of AOE gene expression (with increased AOE activities associated with increased AOE mRNA) could be involved in the pulmonary response to high O_2 exposure. The effects of PTU administration on protecting premature rat pups against O_2-induced lung injury during hyperoxia may be related, at least in part, to PTU's (direct or indirect) effect on enhancing lung AOE gene expression. For certain enzymes, *e.g.* CAT, this enhancing effect appears to depend on a pretranslational mechanism only (CAT mRNA and CAT activity both increase by ~30%). Whereas for other enzymes (*e.g.* CuZnSOD, MnSOD, and GP), the altered gene expression may be more complex following PTU administration and may involve combinations of pretranslational and/or translational/posttranslational mechanisms of control.

PTU is a thioamide derivative that is known to cross the placenta, inhibit thyroid hormone synthesis by blocking the incorporation of iodine, and also inhibit the peripheral deiodination of T_4 to T_3 (1). The consistently decreased serum T_3 and T_4 levels found in the PTU-treated full-term rat pups in our previous study (4) confirm the effectiveness of maternal PTU treatment in reducing thyroid hormone production in the offspring when it is administered in the pregnant/lactating animal's water source. Older studies indicate a protective pulmonary effect of induced hypothyroidism against the oxidant stress of hyperoxia (27) and/or exposure to ozone (28). Interestingly, in addition to PTU's well known antithyroid properties, PTU has recently been reported to be an effective O_2 radical scavenger and to act as an efficient inhibitor of lipid peroxidation (29). It is, in fact, currently being used successfully to treat alcoholic liver disease (30), a condition which has been associated with excess superoxide radical production by hepatic microsomal fractions (31). Thus, it is possible that in addition to enhancing the pulmonary AOE activity responses to hyperoxic challenge, PTU's marked protective effect against O_2 toxicity in the premature rat may also stem from its direct antioxidant properties, its antithyroid properties and its effect on O_2 metabolism, or to a combination of these actions.

Finally, further investigation of the mechanism for the protection of premature rats against O_2-induced lung injury and lethality by PTU treatment will be required before PTU treatment can be considered for protecting the lung of premature infants from toxic damage associated with required high oxygen therapy.

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