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EFFECT OF PROPYLTHIOURACIL TREATMENT ON NADPH-CYTOCHROME P450 REDUCTASE LEVELS, OXYGEN CONSUMPTION AND HYDROXYL RADICAL FORMATION IN LIVER MICROSOMES FROM RATS FED ETHANOL OR ACETONE CHRONICALLY

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Abstract—The antithyroid drug propylthiouracil (PTU) has been shown previously to reduce hepatic oxygen utilization and to protect the liver from ethanol-induced injury. The present study examined the effect of PTU on hepatic microsomal oxygen consumption and on the activities of NADPH-cytochrome P450 reductase (CYP-reductase) and cytochrome P4502E1 (CYP2E1) in rats receiving ethanol or acetone chronically. Liver microsomes from rats treated with ethanol for 29 days displayed increases in (i) O_2 consumption (70%), (ii) hydroxyl radical ($^{\cdot}OH$) production (49%) and (iii) ethanol oxidation (50%). Microsomal CYP2E1 levels were increased markedly by chronic ethanol administration, while CYP-reductase was affected marginally, but not significantly ($P = 0.06$). Chronic treatment with acetone for 14 days, produced similar effects, except that $^{\cdot}OH$ production was not enhanced. Administration of PTU (25 mg/kg/day) to ethanol- or acetone-fed rats, for 10 and 14 days, respectively, led to a marked reduction in the levels and activity of CYP-reductase, and to a decrease in the rates of microsomal O_2 consumption, $^{\cdot}OH$ production and ethanol oxidation, but did not lower the levels of CYP2E1 or the metabolism of the CYP2E1 substrate *N,N*-nitrosodimethylamine. These data suggest that the ability of PTU to protect the liver from ethanol-induced injury may be due to a reduction in the levels of CYP-reductase, thereby minimizing the enhancement of microsomal oxygen consumption and free radical generation associated with ethanol-induced CYP2E1 activity.

Key words: acetone; cytochrome P4502E1; ethanol; NADPH-cytochrome P450 reductase; oxygen-derived free radicals; propylthiouracil

The cytochrome P450-dependent microsomal monoxygenase system is involved in the metabolism of a variety of xenobiotic agents. The hepatic P450 isozyme CYP2E1† is a major component of the ethanol-inducible MEOS [1–3] and has been postulated to play a role in the pathogenesis of ethanol-induced liver injury [4–6]. Some of the proposed mechanisms by which CYP2E1 induction may contribute to ethanol-induced liver injury include oxidative stress due to increased formation of reactive oxygen species [7] and hypoxic damage resulting from increased microsomal oxygen consumption. A possible role for CYP2E1 in promoting intracellular hypoxia is based on the observations that: (i) CYP2E1 displays an exceptionally high degree of oxidase activity, and with NADPH as a cofactor, can reduce oxygen and generate reactive oxygen species in the absence of ethanol or other substrates [7, 8]; and (ii) CYP2E1 is expressed and induced primarily in zone III of the liver acinus [9, 10], a region that is particularly susceptible to ethanol-induced damage [4].

Increases in oxygen consumption and the formation of reactive oxygen species by liver microsomes following chronic ethanol treatment may also result from an elevated content or activity of the flavoprotein CYP-reductase [11–13], which serves to transfer electrons from NADPH and is an obligatory and often rate-limiting component in P450-dependent reactions [14, 15]. Studies using reconstituted membrane systems have shown that in the presence of iron and NADPH, CYP-reductase can undergo auto-oxidation, resulting in the reduction of oxygen to O_2^- and H_2O_2 independently of CYP2E1 [16, 17]. Through Fenton-type and iron-catalyzed Haber–Weiss reactions, O_2^- and H_2O_2 can, in turn, form $^{\cdot}OH$ [16, 17], which is highly reactive and may promote liver injury through oxidative modification of membrane lipids [18, 19], proteins [20] and nucleic acids [21].

The antithyroid drug PTU has been shown to minimize significantly ethanol-induced liver injury

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† Abbreviations: CYP-reductase, NADPH-cytochrome P450 reductase (EC 1.6.2.4); CYP2E1, cytochrome P4502E1; MEOS, microsomal ethanol-oxidizing system; NDMA, *N,N*-nitrosodimethylamine; PTU, propylthiouracil; T_3, triiodothyronine; and T_4, thyroxine.

in a rat model [22] and to reduce markedly the risk of mortality from alcoholic liver disease in humans [23]. The efficacy of PTU in attenuating ethanol-induced liver injury may be explained by the observation that increases in hepatic oxygen utilization as a result of chronic ethanol ingestion in rats can be prevented by the administration of PTU [24–27]. The underlying biochemical mechanisms responsible for the reduction in oxygen consumption by PTU have not been determined. In rats, depletion of thyroid hormones by hypophysectomy [28–30] or more selectively by treatment with the antithyroid drugs methimazole or PTU [28, 31] has been shown to lead to a substantial reduction in hepatic microsomal CYP-reductase activity and mRNA levels. Since CYP-reductase can contribute to microsomal oxygen consumption and the production of reactive oxygen species, the hepatoprotective effect of PTU may be due to its ability to lower CYP-reductase levels and thereby minimize increases in microsomal oxygen consumption and the generation of reactive oxygen species associated with chronic ethanol ingestion. In the present study, we have examined the effect of chronic ethanol and PTU administration on hepatic microsomal CYP-reductase, oxygen consumption, and ·OH formation and on CYP2E1 levels and the microsomal metabolism of the CYP2E1 substrates, ethanol and NDMA. The effect of PTU was investigated further in rats receiving acetone chronically, a procedure that has also been shown to induce hepatic CYP2E1 [32–34].

MATERIALS AND METHODS

Materials. Acetylacetone, cytochrome *c*, NADPH, NDMA, semicarbazide HCl, sodium azide and Tris–HCl were obtained from the Sigma Chemical Co. (St Louis, MO). Acetaldehyde, acetone, DMSO, formaldehyde and sodium EDTA were purchased from BDH Ltd (Poole, U.K.). Chelex-100 metal chelating resin (100–200 mesh) and SDS–polyacrylamide gel were from Bio-Rad Laboratories (Richmond, CA). CYP2E1 and P450-reductase antibodies were from Oxygene (Dallas, TX), and bisacrylamide was from Schwarz-Mann Biotech (Cleveland, OH). All other reagents were of the highest grade commercially available.

Chronic ethanol administration. Female Wistar rats (Charles Rivers, Quebec), weighing 125–135 g, were given continuous access for 29 days to a liquid diet containing ethanol or an isocaloric diet. Two additional groups of ethanol-treated and control rats also received PTU (average dose = 24.3 ± 0.6 mg/kg/day) in the liquid diet during the last 10 days of ethanol treatment. Diets were delivered by an automated four-way feeding system to ensure equal consumption between groups, using a modified version of a technique described by Israel *et al.* [35]. Liquid diets had a total caloric content of 1 kcal/mL with 32% of total calories supplied as fat (a mixture of 80% corn oil, 12% olive oil and 8% cod liver oil), 9% as carbohydrates (maltose dextrin), 23% as protein (casein) and 36% as ethanol or additional carbohydrates. Diets also contained 1.505 g/L vitamin mix No. 20315 (Bioserv, Frenchtown, NJ), 10.03 g/L Bioserv salt mix No. 711, 4 g/L viscarin, 269 mg/L DL-methionine, and 228 mg/L choline chloride.

Chronic acetone administration. Female Wistar rats (175–225 g) were treated for 14 days with either 1% (v/v) acetone in the drinking water or plain tap water. Two additional groups of acetone-treated and control rats concurrently received PTU (25 mg/kg/day) or vehicle (water) by intragastric infusion for 14 days. Rat chow was available *ad lib.* throughout the experiment.

Surgical procedures and isolation of liver microsomes. On the morning of surgery, diets were withheld, and surgery was performed under ether anesthesia. After collecting unheparinized blood samples from the descending aorta, livers were perfused *in situ* with 30 mL of ice-cold phosphate-buffered saline, and then were removed and homogenized in cold 10 mM sodium/potassium phosphate buffer (pH = 7.4) containing 1.14% KCl. Microsomes were separated by centrifugation of the homogenate at 10,000 *g* for 10 min and ultracentrifugation of the supernatant at 100,000 *g* for 60 min. The microsomal pellet was washed once in buffer, resuspended in 2 mL of 50 mM potassium phosphate buffer, and stored at −70° at a protein concentration of approximately 30 mg/ml. In the acetone study, all solutions for liver preparation and biochemical assays were prepared with distilled water treated with Chelex-100 (Bio-Rad) to remove contaminating iron.

Analytical procedures. The levels of T_4 and T_3 were determined in serum by ^{125}I radioimmunoassay (Hormone Research Laboratory, St Michael's Hospital, Toronto). Protein concentrations in microsomal suspensions were measured using Bio-Rad protein assay dye reagent [36] and bovine serum albumin standards. Oxygen consumption was measured using a Clarke oxygen electrode (YSI, Yellow Springs, OH) in incubation mixtures containing 1 mg microsomal protein in 50 mM potassium phosphate buffer (pH 7.4), and 50 μM EDTA. After a 3-min preincubation period, 1 mM NADPH was added to the reaction mixture and oxygen consumption was measured continuously for 3 min at 37°. Total P450 content in liver microsomal suspensions was determined as described previously [37, 38]. CYP-reductase activity was assayed at 30° in 1-mL incubation mixtures consisting of 300 nM potassium phosphate buffer (pH 7.7), 70 nmol cytochrome *c* and 30 μg micosomal protein. Reactions were initiated by the addition of 1 mM NADPH, and the rate of cytochrome *c* reduction was determined spectrophotometrically at 550 nm based on an extinction coefficient of 21 $mM^{-1}cm^{-1}$ [39]. The oxidation of NDMA to formaldehyde was determined as described previously [40]. Reaction mixtures contained 50 mM Tris-HCl buffer (pH 7.4), 150 mM KCl, 10 mM $MgCl_2$, 1 mM NDMA and 1 mg microsomal protein. After a 2-min preincubation period, the reaction was initiated with 0.5 mM NADPH and incubated for 30 min at 37°. The reaction was terminated with 25% (w/v) $ZnSO_4$ and saturated $Ba(OH)_2$. The mixture was centrifuged, and 700 μL of supernatant was combined with 300 μL of concentrated Nash reagent (5 g ammonium acetate

Table 1. Effects of chronic ethanol and PTU treatments on body weight gain and serum levels of thyroid hormones*

	Body weight (g)	Total T_4 (nmol/L)	Total T_3 (nmol/L)
Control diet	180 ± 3	74.1 ± 2.7	1.24 ± 0.07
Ethanol diet	179 ± 4	78.5 ± 5.0†‡	1.47 ± 0.07†‡
Control diet + PTU	180 ± 3	25.0 ± 4.1§‖	0.40 ± 0.04§‖
Ethanol diet + PTU	183 ± 5	28.9 ± 4.7§‖	0.60 ± 0.12§‖

* Rats were fed isocaloric amounts of either ethanol-containing or control liquid diets for 29 days. Two additional groups of ethanol-treated and control rats also received PTU (average dose = 24.3 ± 0.6 mg/kg/day) in the liquid diet for 10 days. Values are means ± SEM (N = 7 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by the *post-hoc* Duncan's Test.

† $P < 0.05$, compared with the PTU-treated control group.
‡ $P < 0.05$, compared with the PTU-treated ethanol group.
§ $P < 0.05$, compared with the control group.
‖ $P < 0.05$, compared with the ethanol-treated group.

and 70 μL acetylacetone in 6 mL of 3% acetic acid) [41]. Following a 30-min incubation at 50° the absorbance at 412 nm was measured and the formaldehyde concentration was determined by comparison with standards of known concentration. Microsomal ethanol oxidation was measured according to the method of Lieber and DeCarli [42] in 25-mL Erlenmeyer flasks with center wells containing 15 mM semicarbazide HCl in 100 mM potassium phosphate buffer. The reaction mixture in the outer well consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM sodium azide (to inhibit catalase present as a contaminant in isolated microsomes), 50 mM ethanol, 5 mM $MgCl_2$, 1 mM sodium EDTA and 3 mg microsomal protein. After a 5-min preincubation, 1 mM NADPH was added, and the vessels were sealed and incubated at 37° for 5 min. The reaction was then terminated with 70% (w/v) trichloroacetic acid. After standing overnight at room temperature, the formation of semicarbazide–acetaldehyde complex was determined by measuring absorbance at 224 nm as compared with standards of known concentration [43]. The production of ·OH and ·OH-like oxidizing species was assayed by measuring the generation of formaldehyde from DMSO [44]. Microsomes (0.25 mg) were suspended in 100 mM potassium phosphate buffer (pH 7.4), 1 mM sodium azide, 25 μM ferric ammonium sulfate: 50 μM EDTA (ferric-EDTA 1:2 chelate) and 50 mM DMSO. After a 2-min preincubation at 37°, the reaction was initiated with 1.5 mM NADPH, terminated after 10 min by the addition of 4% (w/v) trichloroacetic acid, and then centrifuged. To determine formaldehyde concentration, 700 μL of supernatant was combined with 300 μL of concentrated Nash reagent and incubated for 30 min at 50° [41]. The absorbance at 412 nm was measured, and the formaldehyde concentration was determined by comparison with standards of known concentration.

Western blots. Microsomal protein (6 μg) was resolved by electrophoresis in a 7.5% (w/v) SDS–polyacrylamide gel and transferred electrophoretically to nitrocellulose. Immunoblotting was performed with rabbit: anti-rat cytochrome P4502E1 or rabbit: anti-rat NADPH-cytochrome P450 reductase (Oxygene), diluted 1:100, followed by alkaline phosphatase-linked goat: anti-rabbit IgG (diluted 1:1000). The relative density of immunostained bands was quantified by computerized densitometry using a Bio-Rad Imaging Densitometer (No. GS-670) and Bio-Rad Molecular Analyst software.

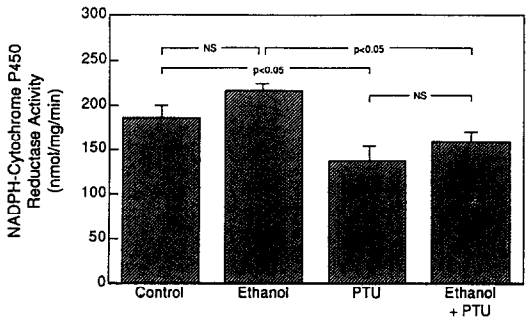

Fig. 1. Effect of PTU on NADPH-cytochrome P450 reductase (CYP-reductase) activity in liver microsomes from ethanol-treated rats. PTU (average dose = 24.3 ± 0.6 mg/kg/day) was administered for 10 days to rats receiving either ethanol diet or control diet for 29 days. CYP-reductase activity was determined by the rate of cytochrome *c* reduction at 500 nm, as described under Materials and Methods. Values are means ± SEM (N = 7 per group). Horizontal bars indicate significant differences between groups, as determined by the *post-hoc* Duncan's Test (cf. Materials and Methods, "Statistical analysis").

Statistical analysis. All values presented are means ± SEM of 6–8 rats per treatment group. Significant differences between groups were determined by two-way analysis of variance followed by the *post-hoc* Duncan's Test. Results were considered significant at $P < 0.05$.

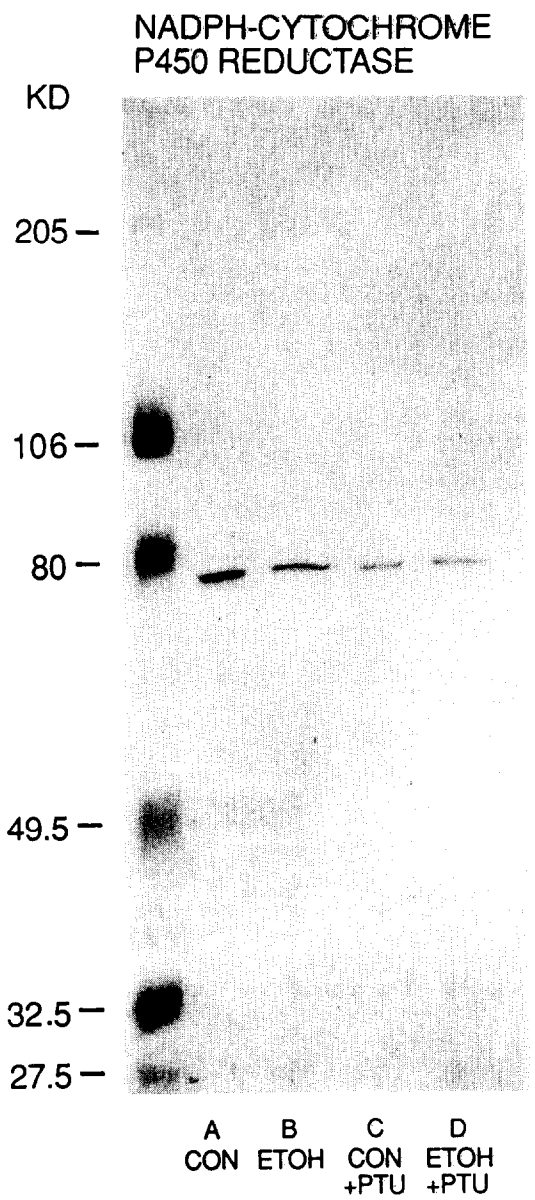

Fig. 2. Immunoblot analysis of NADPH-cytochrome P450 reductase (CYP-reductase) content in liver microsomes isolated from rats receiving (A) control diet, (B) ethanol diet, (C) control diet + PTU, or (D) ethanol diet + PTU (N = 4 per group). The left-hand lane contained prestained protein standards with molecular weights as indicated. Six micrograms of microsomal protein were separated electrophoretically in 7.5% SDS-polyacrylamide gels and immunoblotted with rabbit:anti-rat reductase IgG as described under Materials and Methods. The rabbit:anti-rat CYP-reductase IgG recognized a single 78-kDa protein band corresponding to CYP-reductase.

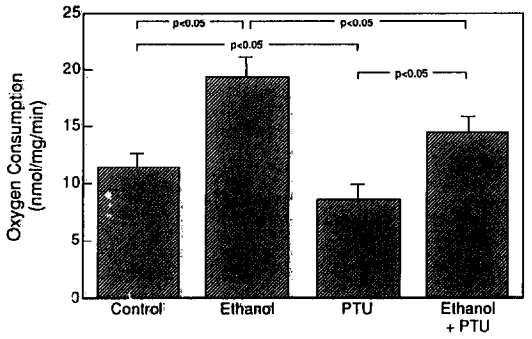

Fig. 3. Effect of chronic ethanol administration and PTU treatments on the rate of NADPH-dependent oxygen consumption in rat liver microsomes. Rats were fed ethanol diet or control diet for 29 days. PTU was administered concurrently during the last 10 days of the experiment. Values are means ± SEM (N = 7 per group). Horizontal bars indicate significant differences between groups, as determined by the *post-hoc* Duncan's Test.

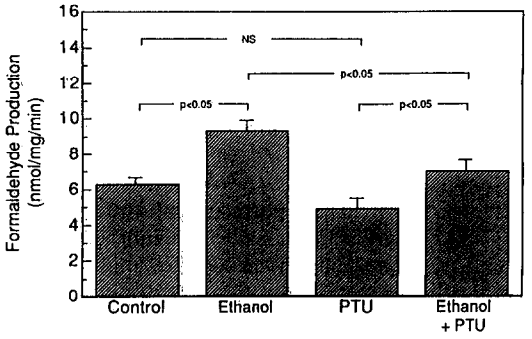

Fig. 4. Effect of chronic ethanol and PTU treatments on the rate of microsomal production of hydroxyl radicals, as determined by the formation of formaldehyde from DMSO in the presence of 1.5 mM NADPH and 25 μM:50 μM ferric-EDTA, as described under Materials and Methods. Values are means ± SEM (N = 7 per group). Horizontal bars indicate significant differences between groups, as determined by the *post-hoc* Duncan's Test.

RESULTS

Chronic ethanol/PTU study. Rats receiving ethanol diets consumed an average of 13.2 ± 0.2 g ethanol/kg/day during the 29-day treatment period. The rate of body weight gain during the course of the experiment was similar between the groups and was not affected by either chronic ethanol treatment or by PTU administration (Table 1). Rats receiving PTU in the liquid diet received an average dose of 24.3 ± 0.6 mg PTU/kg/day. This treatment resulted in a 64% reduction in the levels of circulating T_3 and T_4 (Table 1). The effect of PTU on thyroid hormones was similar in both control and ethanol-treated rats, whereas ethanol treatment itself did not affect T_3 or T_4 levels.

In agreement with prior studies [28–31], induction of hypothyroidism was associated with a decrease in liver microsomal CYP-reductase activity (Fig. 1). Administration of PTU resulted in a 27% decrease in the rate of microsomal cytochrome *c* reduction

Table 2. Effects of ethanol and PTU treatments on hepatic microsomal P450 content and metabolism of ethanol (MEOS) and NDMA*

	Total P450 (nmol/mg)	NDMA (nmol/mg/min)	MEOS (nmol/mg/min)
Control diet	1.00 ± 0.06	1.08 ± 0.04	8.3 ± 0.9
Ethanol diet	1.49 ± 0.09†	2.17 ± 0.09†‡	12.5 ± 1.0†‡§
Control diet + PTU	1.29 ± 0.09†§	1.00 ± 0.03§‖	6.6 ± 0.6§‖
Ethanol diet + PTU	1.57 ± 0.05†‡	2.16 ± 0.08†‡	9.7 ± 1.0‡‖

* Rats were fed isocaloric amounts of either ethanol-containing or control liquid diets for 29 days. Two additional groups of ethanol-treated and control rats also received PTU (average dose = 24.3 ± 0.6 mg/kg/day)' in the liquid diet for 10 days. Values are means ± SEM (N = 7 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by the *post-hoc* Duncan's Test.

† $P < 0.05$, compared with the control group.

‡ $P < 0.05$, compared with the PTU-treated control group.

§ $P < 0.05$, compared with the PTU-treated ethanol group.

‖ $P < 0.05$, compared with the ethanol-treated group.

in both ethanol-treated rats (ethanol diet = 216.2 ± 7.7 nmol/mg/min vs ethanol diet + PTU = 158.1 ± 13.5 nmol/mg/min) and in controls (control diet = 186.0 ± 14.6 nmol/mg/min vs control diet + PTU = 137.2 ± 15.0 nmol/mg/min). CYP-reductase activity in rats treated with ethanol or ethanol + PTU was increased by 15% as compared with the corresponding control groups; however, the effect was not statistically significant ($P = 0.06$).

PTU treatment was also associated with a reduction of CYP-reductase protein levels as determined by western blots. Figure 2 shows a decrease in immunoreactivity of a single 78-kDa protein band recognized by rabbit: anti-rat CYP-reductase IgG in microsomes from PTU-treated rats. In microsomes from control rats treated with PTU, the density of the CYP-reductase immunoreactive band was decreased to 31 ± 15% (N = 4) of the corresponding blot density in untreated rats. In ethanol-fed rats receiving PTU, CYP-reductase immunoblot density was decreased to 34 ± 11% (N = 4) as compared with microsomes from rats receiving ethanol alone. Overall, the effect of PTU was significant only in ethanol-treated animals ($P = 0.05$). CYP-reductase immunoractivity in microsomes from ethanol- and ethanol + PTU-treated rats showed a 46 ± 23 and 53 ± 47% increase, respectively, as compared with their corresponding control groups; however, these effects were not statistically significant.

The rate of hepatic microsomal oxygen consumption (Fig. 3) in rats receiving ethanol for 29 days (19.4 ± 1.8 nmol/mg/min) was 70% higher as compared with rats receiving isocaloric control diets (11.4 ± 0.9 nmol/mg/min). The ethanol-induced increase in oxygen consumption was partially prevented by PTU treatment. Coadministration of PTU reduced the rate of oxygen consumption by 25% in rats receiving either ethanol diet (14.5 ± 1.4 nmol/min/mg) or control diet (8.6 ± 1.3 nmol/min/mg).

The production of ·OH and ·OH-like oxidizing species, as determined by the rate of formaldehyde production from DMSO, was elevated by 49% in microsomes from ethanol-treated rats (Fig. 4). PTU treatment significantly reduced the rate of hydroxyl radical formation in microsomes from ethanol-treated rats (25%).

The effects of chronic ethanol and PTU administration on microsomal P450 content and the metabolism of NDMA and ethanol are summarized in Table 2. Microsomes from ethanol-treated rats showed an increase in total microsomal cytochrome P450 content (48%) and in the rate of ethanol oxidation (50%), and a 2-fold increase in the rate of demethylation of NDMA to formaldehyde. PTU treatment also produced a significant increase in P450 content in microsomes from control rats but not from ethanol-treated rats. While the rate of NDMA metabolism was not altered in microsomes from PTU-treated rats, MEOS activity was reduced by 22% in ethanol-treated rats receiving PTU.

The increase in P450 content and in the rates of NDMA and ethanol oxidation in microsomes from ethanol-treated rats could be accounted for by an increase in CYP2E1 protein levels. Figure 5 shows an increase in immunoreactivity of a 52-kDa protein band recognized by CYP2E1 anti-IgG in microsomes from chronic ethanol-treated rats. Densitometric analysis of CYP2E1 blots revealed a 3-fold increase in the relative intensity of CYP2E1 protein bands from ethanol-treated rat microsomes. CYP2E1 immunoreactivity in microsomes from PTU-treated rats did not differ from controls.

Chronic acetone/PTU study. Since chronic acetone treatment has been shown previously to induce microsomal CYP2E1 [32–34], this regimen was employed to further extend our findings regarding the mechanism of action of PTU on microsomal metabolic activity in rats receiving ethanol chronically. Rats fed 1% (w/v) acetone in the drinking water for 14 days ingested an average daily dose of 90.8 ± 4.4 mg acetone/kg/day. Body weight gain was uniform between groups during the treatment period (Table 3). Chronic ingestion of acetone resulted in hepatomegaly, as indicated by a 14% increase in liver weight. The increase in liver weight did not occur in acetone-treated rats receiving PTU. Intragastric administration of PTU (25 mg/kg/

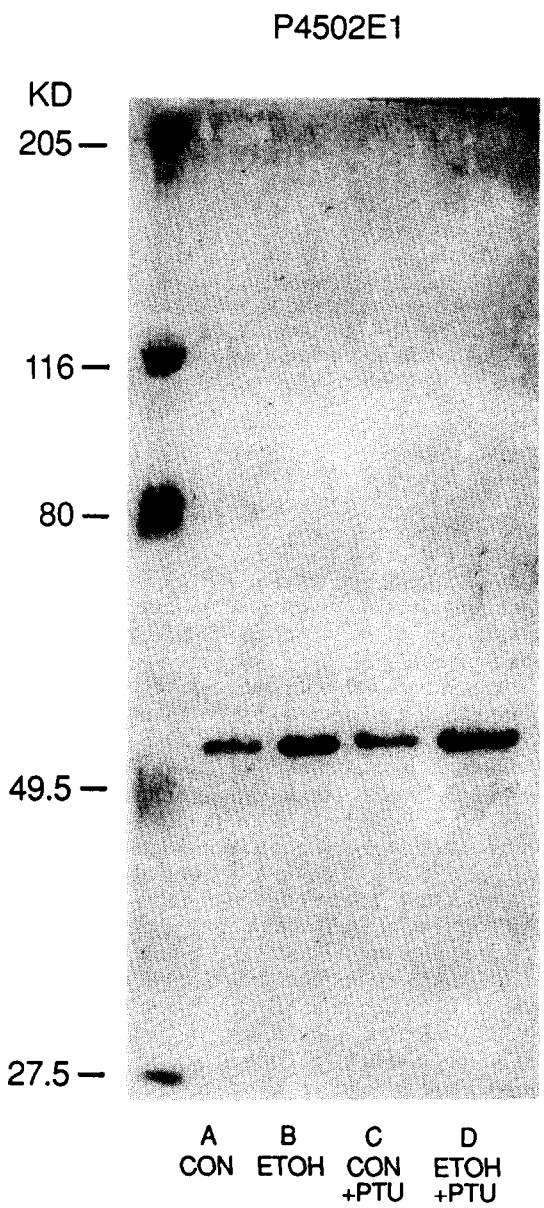

Fig. 5. Immunoblot analysis of CYP2E1 content in liver microsomes isolated from rats receiving (A) control diet, (B) ethanol diet, (C) control diet + PTU, or (D) ethanol diet + PTU (N = 7 per group). The left-hand lane contained prestained protein standards with molecular weights as indicated. Six micrograms of microsomal protein was separated electrophoretically in 7.5% SDS–polyacrylamide gels and immunoblotted with rabbit: anti-rat CYP2E1 IgG, as described under Materials and Methods. The rabbit: anti-rat CYP2E1 IgG recognized a single 52-kDa protein band corresponding to CYP2E1.

day) resulted in a substantial reduction in circulating thyroid hormone levels in both control and acetone-treated rats (Table 3). Serum levels of T_4 and T_3 were reduced by about 75 and 65%, respectively, after 14 days of treatment with PTU, but were unaffected by acetone ingestion.

Associated with the reduction in circulating thyroid hormone levels following PTU treatment was a 40% decrease in microsomal CYP-reductase activity (as measured by the rate of cytochrome *c* reduction), which was observed in microsomes from rats receiving either PTU + acetone or PTU alone (Fig. 6). CYP-reductase activity was not altered significantly by chronic acetone treatment.

Microsomal oxygen consumption was elevated 35% in microsomes from acetone-treated rats (Fig. 7). Administration of PTU significantly reduced the rate of microsomal oxygen consumption in both acetone-treated (21%) and control (32%) rats.

The microsomal generation of ·OH (Fig. 8), as determined by the formation of formaldehyde from DMSO, was not affected by acetone administration, but was decreased significantly by PTU treatment in both control rats (30%) and rats receiving acetone (21%).

The effects of chronic acetone and PTU administration on microsomal P450 content and the metabolism of NDMA and ethanol are summarized in Table 4. Microsomes from acetone-treated rats showed an increase in total microsomal cytochrome P450 content (19%), and in the rate of ethanol oxidation (47%) and a greater than 2-fold increase in the rate of demethylation of NDMA to formaldehyde. PTU treatment also produced a significant increase in total P450 content in microsomes from both control and acetone-treated rats. The rate of microsomal NDMA metabolism was not affected by PTU treatment alone; however, a small but significant increase resulted from PTU administration in rats receiving acetone. MEOS activity was reduced by 32% in acetone-treated rats receiving PTU and by 25% in control rats receiving PTU.

DISCUSSION

The results of this study confirm previous findings that the chronic administration of ethanol in a liquid diet induces hepatic microsomal CYP2E1 in rats [2, 7]. In the present study, treatment with ethanol for 29 days resulted in increased levels of hepatic microsomal P450, a corresponding increase in the levels of CYP2E1 protein, as shown by western blotting, and enhancement of CYP2E1 activity, as evidenced by elevated rates of ethanol oxidation (50%) and NDMA demethylation (100%). Accompanying the induction of CYP2E1 was a 70% increase in the rate of microsomal oxygen consumption and a 49% increase in the rate of ·OH production. In agreement with previous studies [32–34], chronic administration of acetone, a highly specific CYP2E1 substrate, also increased CYP2E1 catalytic activity. As was observed with ethanol, liver microsomes from acetone-treated rats displayed increased rates of oxygen utilization (35%), ethanol oxidation (47%) and NDMA demethylation (146%).

Increased oxygen consumption and oxygen radical formation by microsomes following chronic ethanol treatment are processes that have been implicated previously in promoting ethanol-induced liver injury and may be associated with elevated content or activity of CYP2E1 or CYP-reductase. CYP2E1

Table 3. Effects of chronic acetone and PTU treatments on body weight gain, liver weight and serum thyroid hormone levels*

	Body weight (g)	Liver weight (g)	Total T_4 (nmol/L)	Total T_3 (nmol/L)
Control	241 ± 6	8.5 ± 0.4	62.6 ± 4.8	1.13 ± 0.08
Acetone	245 ± 7	9.7 ± 0.4†‡	53.4 ± 4.2‡§	1.01 ± 0.07‡§
Control + PTU	238 ± 3	8.4 ± 0.3‖	15.1 ± 0.1†‖	0.40 ± 0.03†‖
Acetone + PTU	240 ± 2	8.9 ± 0.3	15.1 ± 0.1†‖	0.38 ± 0.02†‖

* Rats were fed acetone in the drinking water (1%, w/v) for 14 days, while controls received plain tap water only. One group of acetone-treated and control rats also received PTU (25 mg/kg/day) or vehicle by intragastric infusion, concurrently during the 14-day treatment period. Food was available *ad lib*. Values are means ± SEM (N = 8 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by the *post-hoc* Duncan's Test.

† $P < 0.05$, compared with the control group.

‡ $P < 0.05$, compared with the PTU-treated control group.

§ $P < 0.05$, compared with the PTU-treated acetone group.

‖ $P < 0.05$, compared with the acetone-treated group.

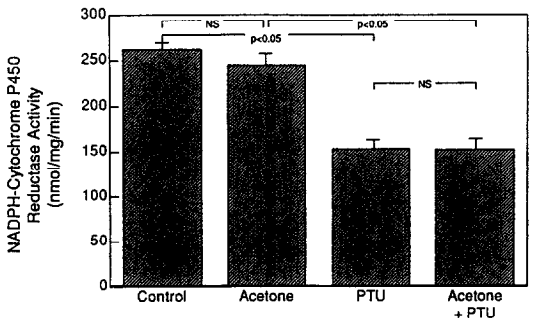

Fig. 6. Effect of PTU on NADPH-cytochrome P450 reductase (CYP-reductase) activity in liver microsomes from acetone-treated rats. PTU (25 mg/kg/day) was administered intragastrically to rats receiving either acetone or plain tap water concurrently for 14 days. CYP-reductase activity was determined by the rate of cytochrome *c* reduction at 550 nm, as described under Materials and Methods. Values are means ± SEM (N = 7 per group). Horizontal bars indicate significant differences between groups, as determined by the *post-hoc* Duncan's Test.

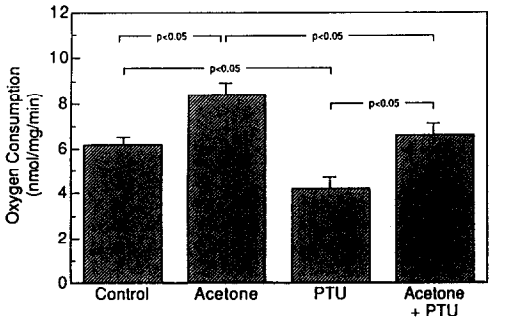

Fig. 7. Effect of chronic acetone and PTU administration on the rate of NADPH-dependent oxygen consumption in rat liver microsomes. Rats were fed acetone or tap water for 14 days. PTU was administered concurrently at a dose of 25 mg/kg/day. Values are means ± SEM (N = 8 per group). Horizontal bars indicate significant differences between groups, as determined by the *post-hoc* Duncan's Test.

displays an exceptionally high degree of NADPH-oxidase activity [8, 45], generating H_2O_2 and H_2O in both the absence and presence of substrates. Estimates derived from *in vitro* studies indicate that CYP2E1 *per se* can contribute significantly to hepatic oxygen consumption [see Refs. 26, 27 and 46]. Consequently, the induction of CYP2E1 oxidase activity by repeated ingestion of ethanol and the resulting increase in microsomal oxygen consumption would cause further depletion of hepatocellular oxygen and may lead to hypoxic injury to pericentral hepatocytes.

Increases in the levels of CYP-reductase may also account for the increase in microsomal oxygen consumption and oxygen radical formation in ethanol-treated rats. There is conflicting evidence regarding the effects of chronic ethanol and acetone treatments on CYP-reductase. While increases in microsomal CYP-reductase activity have been reported in rats receiving chronic ethanol [7, 11–13], other studies have failed to confirm this finding [4, 47]. Similarly, both increases [34, 48] and decreases [32] in CYP-reductase activity have been reported in chronic acetone-treated rats. Under our experimental conditions, chronic treatment with acetone had no effect on microsomal CYP-reductase activity. Microsomes from ethanol-treated rats displayed marginal increases in CYP-reductase activity ($P = 0.06$) and in the levels of CYP-reductase protein, indicating that ethanol may have a slight stimulatory effect on CYP-reductase. Hydroxyl radicals generated through the NADPH-dependent auto-oxidation of CYP-reductase in the presence of iron have been shown to contribute to ethanol oxidation by liver microsomes *in vitro* [16, 49]. In our studies, ferric-EDTA (25 μM : 50 μM) or EDTA (1 mM) was typically included in the reaction

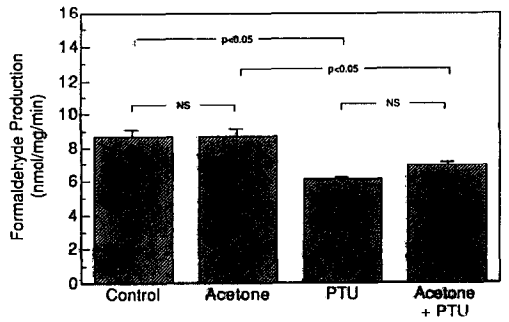

Fig. 8. Effect of chronic acetone and PTU treatments on the rate of microsomal production of hydroxyl radicals determined by the formation of formaldehyde from DMSO in the presence of 1.5 mM NADPH and 25 μM:50 μM ferric-EDTA as described under Materials and Methods. Values are means ± SEM (N = 8 per group). Horizontal bars indicate significant differences between groups, as determined by the *post-hoc* Duncan's Test.

mixtures, which would likely promote the auto-oxidation of CYP-reductase and increase the extent of microsomal ·OH production. Thus, under our experimental conditions, ·OH formation and MEOS activity may be determined largely by CYP-reductase activity. This could account for the finding that ·OH production was increased in ethanol-treated rats, where a marginal increase in CYP-reductase was observed, but was unaffected in acetone-treated rats, where CYP-reductase was clearly unaffected. The heightened contribution of CYP-reductase could also explain why the induction of MEOS activity following ethanol or acetone treatments was low in comparison to the 2 to 3-fold induction of NDMA activity (which is mainly dependent on CYP2E1 activity) and of CYP2E1 immunoreactivity.

In the present study, administration of PTU, which inhibits both thyroid hormone synthesis and peripheral deiodination of T_4 to T_3 [50, 51], reduced serum T_3 and T_4 levels by 64% and the levels and activity of CYP-reductase by 27–34%. This observation is consistent with previous reports that reduction of thyroid hormone levels, by administration of antithyroid drugs such as PTU [31] or methimazole [28], or by surgical manipulations [28–30], lowers microsomal CYP-reductase levels and/or activity. In several respects, the effects of PTU reported here differ from antithyroid manipulations reported previously. A 50% decrease in CYP-reductase activity was observed in hypophysectomized female rats [30], which is greater than the decrease in CYP-reductase observed in PTU-treated rats in our study and is probably attributable to the more complete antithyroid effect of hypophysectomy. In another relevant study by the same group [28], the antithyroid drug methimazole produced a 92% reduction in T_4 levels (to levels near the detection limit) with a corresponding 67% decrease in CYP-reductase activity. In this case, the greater decrease in CYP-reductase activity is probably again attributable to a more potent antithyroid effect of methimazole as compared with PTU.

Although PTU treatment produced a slight increase in microsomal P450 content, no effect was observed on CYP2E1 levels or catalytic activity, as demonstrated by western blots and rates of NDMA demethylation, respectively. The possible identity of the other P450 isozymes induced by PTU was not examined in the present study; however, the steroid hydroxylases CYP3A2 and CYP2A2 are likely candidates [30]. In previous studies, hypophysectomy was shown to increase the hepatic expression of CYP3A2, an effect that is abolished by T_4 replacement [52], and to increase the levels of CYP3A2 and CYP2A2 protein [30].

PTU has been employed previously in both clinical [23] and experimental [22] studies to revert or to prevent ethanol-induced liver injury. The results of this study implicate several mechanisms that may contribute to the hepatoprotective effect of PTU.

Table 4. Effects of acetone and PTU treatments on hepatic microsomal P450 content and metabolism of ethanol (MEOS) and NDMA*

	Total P450 (nmol/mg)	NDMA (nmol/mg/min)	MEOS (nmol/mg/min)
Control	1.22 ± 0.05	0.96 ± 0.03	13.3 ± 0.4
Acetone	1.45 ± 0.06†‡	2.36 ± 0.13†‡§	19.6 ± 0.6†‡§
Control + PTU	1.46 ± 0.11†‡	1.10 ± 0.07‡‖	9.0 ± 0.8†‡§
Acetone + PTU	1.75 ± 0.05†§‖	2.79 ± 0.19†§‖	15.7 ± 1.0§‖

* Rats were fed acetone in the drinking water (1%, w/v) for 14 days, while controls received plain tap water only. One group of acetone-treated and control rats also received PTU (25 mg/kg/day) or vehicle by intragastric infusion, concurrently during the 14-day treatment period. Food was available *ad lib*. Values are means ± SEM (N = 8 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by the *post-hoc* Duncan's Test.

† $P < 0.05$, compared with the control group.

‡ $P < 0.05$, compared with the PTU-treated acetone group.

§ $P < 0.05$, compared with the PTU-treated control group.

‖ $P < 0.05$, compared with the acetone-treated group.

First, PTU was found to offset the increase in microsomal oxygen consumption associated with CYP2E1 induction in ethanol- and acetone-treated rats. This observation is consistent with studies indicating that PTU administration in chronic ethanol-fed rats reduces hepatic oxygen utilization both in isolated perfused livers [25–27], and in the intact animal [24]. Second, in contrast to ethanol, which increased ·OH production, PTU treatment reduced the rate of ·OH formation in ethanol- and acetone-treated rats. Lastly, microsomal oxidation of ethanol to acetaldehyde, which was increased substantially following treatment with ethanol or acetone, was reduced greatly in microsomes from PTU-treated rats.

The observed reduction in microsomal oxygen consumption, ·OH production and ethanol oxidation in PTU-treated rats appears to result from a decrease in the levels and activity of CYP-reductase. This may have a dual effect involving either a reduction in electron transport for CYP2E1 oxidase reactions or, alternatively, a decrease in the iron-catalyzed auto-oxidation of CYP-reductase, occurring independently of CYP2E1. Our results indicating a decreased rate of ethanol oxidation in PTU-treated rats, in the absence of changes in CYP2E1 levels or catalytic activity, can be explained by the reduction in iron-catalyzed ·OH production, due to the lower levels and activity of CYP-reductase. The role of iron in these reactions is complex and depends on the chelated form of iron utilized, the concentration used, and the oxygen radical being investigated [53]. Although our system contained EDTA and trace amounts of iron, it is possible that similar conditions may be reached in the physiological state. Previous studies have shown that both ferric-ATP and ferric-citrate, which are more likely to exist *in vivo*, are also capable of promoting ·OH production and lipoperoxidation [16]. *In vitro*, microsomal iron levels as low as 1 μM are sufficient to catalyze Haber–Weiss reactions leading to NADPH-dependent ·OH formation by CYP-reductase [17]. The extent to which CYP-reductase contributes to hepatic oxygen consumption, oxygen radical generation, and ethanol oxidation *in vivo* is unknown, but it may be significant under conditions in which liver iron levels are elevated, as described in chronic ethanol-fed rats [13] and in human alcoholics with liver disease [54, 55]. This view is in line with recent findings that coadministration of iron in rats receiving chronic ethanol treatment hastens the onset and increases the severity of ethanol-induced liver injury [56, 57].

In summary, these results indicate that chronic ethanol ingestion results in the induction of CYP2E1 and enhances microsomal oxygen utilization, ·OH production and ethanol oxidation, processes that may be involved in promoting ethanol-induced liver pathologies. The ability of PTU to minimize these effects by lowering CYP-reductase levels may contribute to its therapeutic efficacy in reducing liver injury.

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