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CATALASE-DEPENDENT ETHANOL METABOLISM *IN VIVO* IN DEERMICE LACKING ALCOHOL DEHYDROGENASE

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Abstract—Pathways of ethanol elimination in alcohol dehydrogenase (ADH)-positive and -negative deermice were studied using the catalase inhibitor, 3-amino-1,2,4-triazole. To verify that aminotriazole inhibited catalase effectively, the characteristic decrease in catalase-H_2O_2 which occurs in saline-treated controls when ethanol is peroxidized was monitored at 660–640 nm in perfused deermouse livers. Following 1.5 hr of pretreatment with aminotriazole (1.5 g/kg), the peroxidatic activity of catalase measured *in vitro* was inhibited by greater than 99%. Under these conditions, ethanol did not decrease catalase-H_2O_2 in perfused livers, indicating that catalase was inhibited. Ethanol and aniline oxidation by microsomes were also inhibited by about 67–90% after 1.5 hr of pretreatment with aminotriazole. In ADH-positive deermice, pretreatment with aminotriazole for 1.5 hr prior to injection of ethanol (2.0 g/kg) decreased rates of ethanol elimination *in vivo* from 13.2 ± 0.8 to 10.2 ± 0.4 mmoles/kg/hr. In ADH-negative deermice, similar treatment decreased rates of ethanol elimination *in vivo* from 4.5 ± 0.4 to 1.1 ± 0.6 mmoles/kg/hr. Following pretreatment with aminotriazole (1.0 g/kg) for 6 hr, rates of ethanol elimination in ADH-negative deermice returned to near basal values. Under these conditions, the peroxidatic activity of catalase measured *in vitro* and the ethanol-dependent decrease in catalase-H_2O_2 in perfused livers also returned to near basal levels; however, the oxidation of ethanol by cytochrome P-450 was inhibited completely. It is concluded, therefore, that time of pretreatment with aminotriazole is an important variable which must be controlled carefully to inhibit catalase completely. Since catalase was active while cytochrome P-450 was not following 6 hr of pretreatment with aminotriazole, it is concluded that ethanol elimination occurs predominantly via catalase-H_2O_2 in ADH-negative deermice under these conditions.

Two pathways of ethanol oxidation, catalase-H_2O_2 and cytochrome P-450, have been described in addition to alcohol dehydrogenase (ADH); however, their quantitative contributions to ethanol metabolism *in vivo* remain unclear. The oxidation of ethanol via catalase was described first by Keilin and Hartree in 1945 [1]. Half-maximal rates occur with ethanol concentrations around 15 mM [2, 3], and the rate of the reaction is limited by the supply of H_2O_2 [4]. Substrates for peroxisomal H_2O_2 generating flavoproteins (i.e. urate and glycolate) have been shown to increase rates of catalase-dependent peroxidation of ethanol significantly in the perfused liver [5]. More recently, it has been shown that H_2O_2 production from the β-oxidation of fatty acids by peroxisomes can also stimulate catalase-dependent ethanol metabolism [6]. In addition, a microsomal ethanol-oxidizing system described first by Orme-Johnson and Ziegler [7] has been studied extensively by Lieber and colleagues [8, 9]. This system has a K_m for ethanol of 7–12 mM, it requires O_2 and NADPH, and is inhibited partially by CO. A form of cytochrome P-450 with a high affinity for ethanol and aniline, and which is induced by ethanol, acetone and imidazole, has been isolated in pure form from rabbit liver by Koop *et al.* [10, 11].

A mutant strain of deermouse, *Peromyscus maniculatus*, deficient in ADH [12, 13] has been shown by Burnett and Felder [14] to eliminate ethanol at rates up to 60% as fast as deermice which have ADH. Thus, the ADH-negative deermouse should be an ideal model in which to study non-ADH-dependent pathways of ethanol metabolism without the use of inhibitors of ADH (e.g. alkyl pyrazoles). One such study was performed by Shigeta *et al.* [15]. They concluded that catalase does not participate significantly in ethanol metabolism in ADH-negative deermice because the catalase inhibitor, aminotriazole, did not diminish rates of ethanol elimination *in vivo* even though the catalatic activity of catalase was decreased 90–95%. However, the effect of aminotriazole on the peroxidatic activity of catalase was not measured in their study.

The purpose of these experiments was to measure ethanol elimination in ADH-negative deermice under conditions where catalase-dependent peroxidation of ethanol was inhibited completely in a verifiable manner by aminotriazole.

MATERIALS AND METHODS

Animals. Deermice with normal hepatic ADH (genotype ADH^F/ADH^F; ADH-positive) and deermice genetically deficient in ADH (ADH^N/ADH^N; ADH-negative) were obtained from a breeding colony maintained at the University of North Carolina at Chapel Hill which was established from breeding pairs supplied by Dr. M. R. Felder of the University

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of South Carolina. Deermice were fed chow diet and tap water *ad lib.* and were housed in a room with a 16-hr light and 8-hr dark cycle.

Ethanol elimination in vivo. Animals were injected with 2.0 g/kg ethanol in saline i.p. and breath samples were taken every 30 min for 3–5 hr as described previously [16]. Ethanol was quantitated chromatographically by injecting 1-ml breath samples onto a carbowax 60/80 column and analyzing for ethanol with a flame ionization detector. Operating parameters were: oven, 110°, detector, 250°; injection port, 250°; and carrier gas flow, 40 ml/min. Breath ethanol values were converted into blood ethanol concentrations and rates of blood ethanol elimination were calculated from the linear decline in concentration over time [16]. Animals were injected with aminotriazole 1.5 hr (1.5 g/kg, i.p.) or 6 hr (1.0 g/kg) prior to ethanol. The latter conditions were chosen to duplicate conditions employed by Shigeta *et al.* [15] precisely.

Liver perfusion. Livers from deermice were perfused in a non-recirculating system by a modification of a rat liver perfusion system routine in this laboratory [17]. The perfusate was Krebs–Henseleit buffer (pH 7.4, 37°) saturated with a mixture of O_2:CO_2 (19:1). Deermice were anesthetized with pentobarbital (80 mg/kg), the abdomen was opened, and the portal vein was cannulated. The vena cava was then severed and the liver was perfused at flow rates of 6–8 ml/min/g. Under similar conditions, oxygen uptake was constant and lactate dehydrogenase release was minimal over a 2-hr period [18]. The steady-state level of catalase–H_2O_2 was determined spectrophotometrically (660–640 nm) through a lobe of the liver as described by Sies and Chance [19].

Catalatic and peroxidatic activities of catalase in vitro. Hydrogen peroxide forms an intermediate with catalase, catalase–H_2O_2. Catalase–H_2O_2 may then undergo subsequent reactions with another molecule of H_2O_2 to generate oxygen and water (the catalatic reaction) or with hydrogen donors such as methanol or ethanol to generate water and the corresponding peroxidized product (the peroxidatic reaction). To study the effect of aminotriazole on the catalatic and peroxidatic activities of catalase, ADH-negative deermice were injected with aminotriazole as described above. After 1.5 or 6 hr of pretreatment, deermice were anesthetized and their livers perfused briefly with Krebs–Henseleit buffer. Livers were then homogenized in 4 vol. of ice-cold sucrose (0.25 M, pH 7.4) and kept on ice. Homogenate equivalent to 5 mg of tissue was incubated in 1.0 ml of phosphate buffer (0.2 M, pH 7.4, 37°) containing 10 mM H_2O_2, and the increase in O_2 concentration was monitored polarographically with a Teflon-shielded, Clark-type O_2 electrode. Peroxidatic activity of catalase was measured in 1.0 ml of the same incubation mixture, except that semicarbazide (15 mM) and methanol (100 mM) were included, and homogenate equivalent to 200 mg of tissue was added per incubation. The reaction was stopped after 30 sec with 0.1 ml of 40% trichloroacetic acid and formaldehyde was measured colorimetrically as described previously [20].

Preparation of microsomes. Deermice were killed by decapitation and their livers were removed quickly. Livers were homogenized in ice-cold buffer consisting of 100 mM Tris Cl, 150 mM KCl, 1 mM EDTA, pH 7.4, and the microsomal fraction was isolated by differential centrifugation. The initial 100,000 *g* pellet was resuspended in 100 mM sodium pyrophosphate, 1 mM EDTA, pH 7.4, and centrifuged again at 100,000 *g* for 1 hr. Washed pellets were resuspended in 1.15% KCl. Protein content was determined with the Biuret method [21].

Microsomal oxidation of ethanol and aniline. The oxidation of ethanol by deermouse microsomes was monitored by measuring the production of acetaldehyde employing methods described previously [3]. Briefly, 1–2 mg of microsomal protein was incubated in capped vessels in 80 mM phosphate buffer, pH 7.4, containing 20 mM nicotinamide, 1 mM azide to inhibit catalase, and 10 mM $MgCl_2$. Ethanol (100 mM) was added, and reactions were initiated by the addition of an NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate and 200 mU/ml isocitrate dehydrogenase. Incubations were carried out for 10 min and were terminated by the addition of 0.2 ml of 40% trichloroacetic acid. The acetaldehyde formed was trapped as the semicarbazone by diffusion into a center well containing 15 mM semicarbazide in 160 mM phosphate buffer, pH 7.0. The acetaldehyde semicarbazone was quantitated spectrophotometrically at 224 nm employing appropriate acetaldehyde standards. Deferoxamine (0.5 mM), an iron-trapping agent, had no effect upon rates of ethanol oxidation, indicating that the oxidation of ethanol by free radicals was minimal under these conditions.

The hydroxylation of aniline was monitored by measuring the formation of *p*-aminophenol colorimetrically [22]. Microsomes were incubated in the reaction mixture described above but containing 2.5 mM aniline instead of ethanol. After 10-min incubations at 37°, reactions were terminated with trichloroacetic acid, and samples were centrifuged and neutralized. The supernatant fraction was assayed for *p*-aminophenol as described elsewhere [22].

Statistical analysis. Differences between control and experimental groups were assessed using Student's *t*-test [23].

RESULTS

Effect of ethanol on catalase–H_2O_2. The reaction of one mole of ethanol with one mole of catalase–H_2O_2 results in the formation of one mole of acetaldehyde and the destruction of the catalase–H_2O_2 complex. Thus, ethanol decreases the steady-state level of catalase–H_2O_2 in the liver [19]. In livers from ADH-negative deermice given saline vehicle 1.5 or 6 hr before perfusion, ethanol decreased the steady-state level of catalase–H_2O_2 (Figs. 1 and 2) due to peroxidation of ethanol via catalase–H_2O_2 as expected. Further, when livers from ADH-negative deermice pretreated for 6 hr with aminotriazole were perfused, ethanol also decreased catalase–H_2O_2 (Fig. 1), providing qualitative evidence that pretreatment with aminotriazole for 6 hr did not inhibit the peroxidatic function of catalase. In contrast, when aminotriazole was injected 90 min before perfusion, etha-

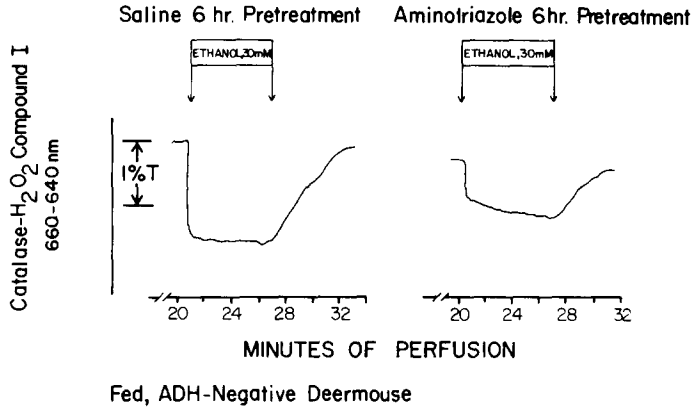

Fig. 1. Ethanol-induced changes in catalase–H_2O_2 after 6 hr of pretreatment with aminotriazole. Livers from ADH-negative deermice were perfused in a non-recirculating system, and the steady-state level of catalase–H_2O_2 was monitored spectrophotometrically as described in Materials and Methods. Aminotriazole was included in the perfusate at a concentration of 11.8 mM, and ethanol was infused with a precision infusion pump as indicated by the horizontal bars and arrows. Left panel: liver from an ADH-negative deermouse given saline 6 hr before perfusion. Right panel: liver from an ADH-negative deermouse given aminotriazole (1.0 g/kg) 6 hr before perfusion. Typical experiments.

nol did not affect the 660–640 nm signal (Fig. 2), indicating that catalase was inhibited effectively. Similar results were obtained in livers from ADH-positive deermice (data not shown).

Catalatic and peroxidatic activities of catalase in ADH-negative deermice after treatment with aminotriazole. Pretreatment of ADH-negative deermice with aminotriazole for 1.5 hr decreased the catalatic activity of catalase by 87% (Table 1). This treatment also decreased the peroxidatic activity of catalase to less than 1% of control levels. In contrast, after 6 hr of pretreatment with aminotriazole, catalatic activity returned to 53% of control values. Under these conditions, the peroxidatic activity of catalase returned to near basal levels (Table 1).

Oxidation of ethanol and aniline by microsomes from ADH-negative deermice after treatment with aminotriazole. Pretreatment of ADH-negative deermice with aminotriazole for 1.5 hr decreased the oxidation of ethanol by 85% from 12 ± 1 to 2 ± 1 nmoles/min/mg protein (Table 2). Hydroxylation of aniline, which is catalyzed predominantly by the same isozyme of cytochrome P-450 as ethanol (D. R. Koop, J. A. Handler, M. F. Felder, M. J. Coon and R. G. Thurman unpublished), was inhibited by 67% at this time point. After 6 hr of pretreatment with aminotriazole, oxidation of ethanol by microsomal cytochrome P-450 was undetectable and aniline hydroxylation was decreased by over 90% (Table 2).

Blood ethanol elimination in vivo. Rates of ethanol elimination by ADH-positive deermice of 13.2 ± 0.8 mmoles/kg/hr were decreased by 24% following pretreatment with aminotriazole for 1.5 hr

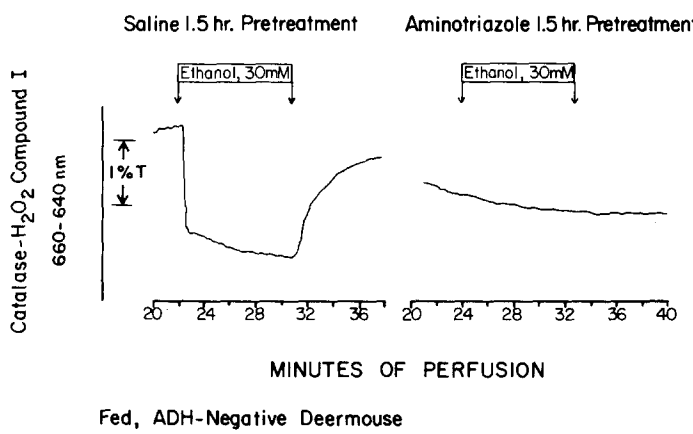

Fig. 2. Ethanol-induced changes in catalase–H_2O_2 after pretreatment for 1.5 hr with aminotriazole. Livers from ADH-negative deermice were perfused as described in Fig. 1. Left panel: liver from an ADH-negative deermouse given saline 1.5 hr before perfusion. Right panel: liver from an ADH-negative deermouse given aminotriazole (1.5 g/kg) 1.5 hr before perfusion. Typical experiments.

Table 1. Time course of inhibition of the catalatic and peroxidatic activities of catalase by 3-amino-1,2,4-triazole in ADH-negative deermice

Treatment	Catalatic activity (mmoles/g/hr)	(% of control)	Peroxidatic activity (μmoles/g/hr)	(% of control)
Saline	12.1 ± 1.0	100	493 ± 10	100
Aminotriazole, 1.5 hr	1.5 ± 0.2*	13	<2*	<1
Aminotriazole, 6 hr	5.7 ± 0.1*	53	468 ± 19	95

ADH-negative deermice were treated with aminotriazole for various time intervals as described in Materials and Methods. At appropriate times, deermice were anesthetized and their livers were removed and homogenized in ice-cold sucrose (0.25 M, pH 7.4). Catalatic (O_2 production from H_2O_2) and peroxidatic activities (methanol oxidation) of catalase were measured as described in Materials and Methods. Data are mean ± SEM for four livers per group.

* $P < 0.001$, compared with appropriate saline control values.

($P < 0.05$, Table 3). Therefore, metabolism via ADH predominates in the ADH-positive deermouse under these conditions. In untreated ADH-negative deermice, rates of ethanol elimination in this study were 4.5 ± 0.4 mmoles/kg/hr (Table 3). Pretreatment for 1.5 hr with aminotriazole decreased rates by about 75% to 1.1 ± 0.6 mmoles/kg/hr (Table 3). In contrast, rates of ethanol elimination in ADH-negative deermice returned to near basal levels following pretreatment for 6 hr with aminotriazole (Table 3).

DISCUSSION

The quantitative roles of catalase–H_2O_2 and cytochrome P-450 in overall hepatic ethanol oxidation have been controversial. The availability of the ADH-negative deermouse [12, 13] provides a model in which to study these pathways without the use of inhibitors of ADH which may have nonspecific effects [24]. Several studies have addressed pathways of ethanol elimination in the ADH-negative deermouse [15, 24]. Takagi *et al.* [24] concluded that ethanol oxidation occurs predominantly via cytochrome P-450 in ADH-negative deermice by using reported isotope effects for catalase and cytochrome P-450 from rat liver [25]. However, isotope effects vary between experimental systems [25] and species [26]. In fact, isotope effects in microsomes from ADH-negative deermice are actually 4-fold greater than published values from rat liver (M. Ingelman-Sundberg, personal communication). Therefore, caution must be used in interpreting results of experiments employing isotope effects. Shigeta *et al.* [15] also concluded that catalase does not play a significant role in ethanol metabolism in ADH-negative deermice based on the observation that a 6-hr pretreatment with aminotriazole did not diminish rates of ethanol elimination in ADH-negative deermice *in vivo*. This conclusion must be reconsidered, however, in view of the results presented in this study (see below).

After 1.5 hr of pretreatment with aminotriazole, the peroxidatic activity of catalase was inhibited fully (Table 1). This conclusion is based on the observations that there were no changes in the steady-state level of catalase–H_2O_2 upon infusion of ethanol in perfused liver (Fig. 2), and that the peroxidatic activity of catalase measured *in vitro* was undetectable (Table 1). Concomitantly, rates of ethanol elimination by ADH-negative deermice were diminished by about 75% (Table 3). Cytochrome P-450-dependent activities were also inhibited 80–90% after 1.5 hr of pretreatment with aminotriazole (Table 2); therefore, it is not possible to determine from these data whether catalase or cytochrome P-450 is the predominant pathway of ethanol oxidation in this mutant strain following 1.5 hr of pretreatment with aminotriazole. Since the ADH-negative deermouse still eliminated ethanol at about 25% of the basal rate after aminotriazole treatment (Table 3), we conclude that residual elimination is via non-metabolic physiological processes. In support of this argument, Shigeta *et al.* [15] concluded that 10–15% of ethanol is eliminated via non-metabolic routes in the ADH-negative strain.

After 6 hr of pretreatment with aminotriazole, the steady-state level of catalase–H_2O_2 in perfused livers

Table 2. Time course of inhibition of cytochrome P-450-dependent ethanol oxidation and aniline hydroxylation by 3-amino-1,2,4-triazole in ADH-negative deermice

Treatment	Ethanol oxidation (nmoles/min/mg)	(% of control)	Aniline hydroxylation (nmoles/min/mg)	(% of control)
Saline	11.9 ± 1.2	100	1.7 ± 0.2	100
Aminotriazole, 1.5 hr	1.8 ± 1.1*	15	0.6 ± 0.2*	33
Aminotriazole, 6 hr	<0.4*	<3	0.2 ± 0.1*	10

ADH-negative deermice were treated with aminotriazole as described in Table 1. At appropriate times, deermice were decapitated, and their livers were removed. Microsomes were isolated by differential centrifugation. Rates of oxidation of ethanol and aniline were determined as described in Materials and Methods. Data are mean ± SEM for four livers per group.

* $P < 0.001$, compared with appropriate saline control values.

Table 3. Effect of 3-amino-1,2,4-triazole on ethanol elimination by deermice

	Treatment	Ethanol elimination (mmoles/kg/hr)
ADH-positive	Saline, 1.5 hr	13.2 ± 0.8
	Aminotriazole, 1.5 hr	10.2 ± 0.4*
ADH-negative	Saline, 6 hr	4.0 ± 0.8
	Aminotriazole, 6 hr	3.4 ± 0.7
ADH-negative	Saline, 1.5 hr	4.5 ± 0.4
	Aminotriazole, 1.5 hr	1.1 ± 0.6†

ADH-positive and ADH-negative deermice received 2.0 g/kg ethanol i.p., and ethanol elimination was measured as described in Materials and Methods. Aminotriazole was injected at 1.5 (1.5 g/kg, i.p.) or 6 hr (1.0 g/kg, i.p) before ethanol as described in Materials and Methods. In studies where aminotriazole was given 1.5 hr before ethanol, deermice also received 1.5 g/kg aminotriazole i.p. 60–90 min after injection of ethanol. Data are mean ± SEM for three to seven animals per group.

* $P < 0.05$, compared with appropriate saline control value.

† $P < 0.01$, compared with appropriate saline control value.

was decreased by ethanol (Fig. 1). In addition, the peroxidatic activity of catalase measured *in vitro* returned to basal levels (Table 1). Therefore, it is concluded that the ADH-negative deermouse oxidizes ethanol via catalase–H_2O_2 following 6 hr of treatment with aminotriazole. In the rat, catalase activity recovers by about 50% as soon as 3 hr after injection of aminotriazole [27]. In contrast, the oxidation of ethanol by cytochrome P-450 was undetectable after 6 hr of pretreatment with aminotriazole (Table 2). The fact that catalase-dependent peroxidatic activity returned to basal values in 6 hr after aminotriazole yet cytochrome-P-450-dependent ethanol oxidation remained depressed is most likely due to the relative rates of synthesis of these two hemoproteins. Total hepatic catalase activity can be resynthesized in about 12 hr [27], whereas the turnover of cytochrome P-450 is much slower (*ca.* 40 hr, Ref. 28). The oxidation of several substrates for cytochrome P-450 has also been reported to be depressed after 6 hr of pretreatment with aminotriazole [29]. Since ethanol elimination and the peroxidatic activity of catalase returned to near basal levels yet cytochrome P-450-dependent oxidation of ethanol remained depressed after 6 hr of aminotriazole (Fig. 3), it is concluded that ethanol oxidation is catalyzed exclusively by catalase in ADH-negative deermice under these conditions. The con-

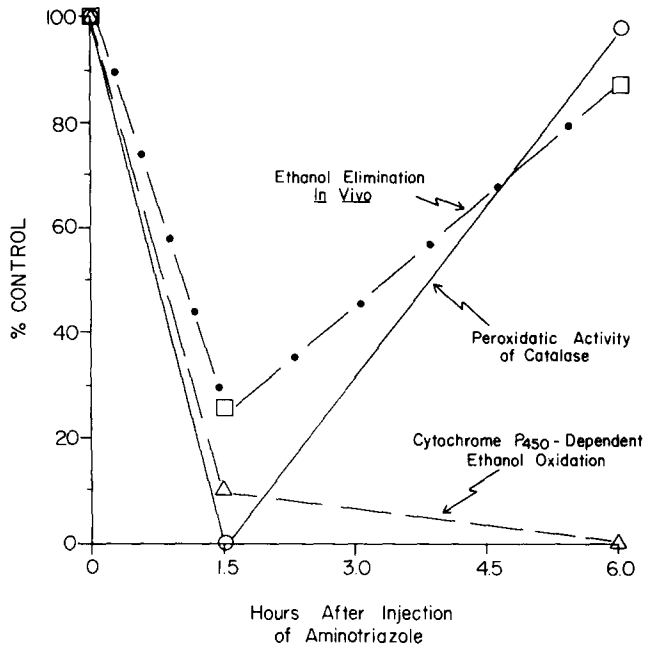

Fig. 3. Temporal effects of aminotriazole on ethanol elimination and pathways of ethanol oxidation in ADH-negative deermice. Key: (□) ethanol elimination *in vivo*; (○) the peroxidatic activity of catalase; and (△) the oxidation of ethanol by microsomes. Data from Tables 1–3.

clusion of Shigeta *et al.* [15] that ethanol oxidation by the ADH-negative deermouse is carried out predominantly by cytochrome P-450 is inconsistent with the data in this report. These studies point out that caution must be used when aminotriazole is employed to inhibit catalase in order to study pathways of ethanol metabolism. However, by monitoring effects of aminotriazole on catalase, cytochrome P-450, and ethanol elimination *in vivo* carefully, it is possible to conclude that ethanol elimination proceeds exclusively via catalase–H_2O_2 in the ADH-negative deermouse following 6 hr of pretreatment with aminotriazole. More work will be necessary in the future to determine the relative quantitative contributions of catalase and cytochrome P-450 to ethanol elimination unequivocally in the absence of metabolic inhibitors.

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