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DIFFERENTIAL EFFECTS OF THE CYTOCHROME P-450/REDUCTASE RATIO ON THE OXIDATION OF ETHANOL AND THE HYDROXYL RADICAL SCAVENGING AGENT 2-KETO-4-THIOMETHYLBUTYRIC ACID (KMBA)*

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Abstract—NADPH–cytochrome P-450 reductase catalyzes a low rate of oxidation of hydroxyl radical scavenging agents such as ethanol and 2-keto-4-thiomethylbutyric acid (KMBA), in a reaction markedly stimulated by the addition of ferric-EDTA. The effect of various ratios of cytochrome P-450 (phenobarbital-inducible isozyme)/reductase on the oxidation of ethanol and KMBA was determined. There was essentially no increase in KMBA oxidation over the range of ratios from 0.5 to 5 as compared to the reductase-catalyzed rate. High ratios actually caused some decrease in KMBA oxidation, which was especially notable under conditions of increased rates of hydroxyl radical generation (presence of increasing amounts of ferric-EDTA). This decrease at high P-450/reductase ratios could reflect tight coupling of reductase to P-450-PB, therefore decreasing electron transfer from reductase to ferric-EDTA, or could involve non-specific scavenging of ·OH by P-450-PB. Indeed, native, but not boiled, P-450 inhibited KMBA oxidation by the xanthine oxidase system. By contrast, the oxidation of ethanol was stimulated at all concentrations of P-450-PB, and this increase was not sensitive to desferrioxamine. However, under conditions of high rates of ·OH production, the ethanol oxidation profile tended to resemble the KMBA oxidation profile with respect to the effect of P-450-PB, whereas the two profiles were different under conditions of low rates of ·OH production. These results suggest that P-450-PB does not catalyze the oxidation of ·OH scavengers or promote the production of ·OH, even at ratios of P-450/reductase approaching those found with intact microsomes and even in the presence of excess iron-EDTA, whereas ethanol is directly oxidized by P-450-PB, as are typical drug substrates. However, the P-450-PB-dependent oxidation of ethanol can be masked under conditions in which ·OH production is increased.

Previous studies have shown that the oxidation of a variety of hydroxyl radical (·OH)‖ scavenging agents by isolated rat liver microsomes and by purified components of the microsomal mixed-function oxidase system is dependent upon the amount of iron-EDTA in the system [1, 2]. It was noted that the presence of NADPH–cytochrome P-450 reductase is sufficient to promote the oxidation of ·OH scavengers, and that addition of cytochrome P-450 purified from phenobarbital-treated rats (P-450-PB) has no effect on the reductase-mediated rates [2]. Electron spin resonance studies with the spin-trapping agent 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) detected a characteristic DMPO-·OH signal when iron-EDTA was added to microsomes or to the reductase purified from phenobarbital-treated rats [3, 4]. Ferric-EDTA stimulates oxidation of NADPH by the reductase [5, 6], and the increase in ·OH production produced by ferric-EDTA is not sensitive to superoxide dismutase [7]. These results suggested that ferric-EDTA could be reduced directly by the reductase, and the resulting ferrous-EDTA reacted with H_2O_2 produced during microsomal electron transfer or autoxidation of ferrous ion, to generate ·OH.

One ·OH scavenging agent, ethanol, displayed different reactivities in the microsomal and the reconstituted systems. Unlike other ·OH scavengers, ethanol oxidation could be catalyzed by organic hydroperoxides in the absence of NADPH [8]; ethanol oxidation occurs in the presence of concentrations of the potent iron chelating agent, desferrioxamine, which strongly blocks the oxidation of other ·OH scavengers [1]; and ethanol oxidation increases linearly upon the addition of cytochrome P-450-PB to the reductase system [9]. Thus, ethanol has some properties of typical ·OH scavengers, yet, also behaves as a substrate for direct oxidation by cytochrome P-450. In recent experiments utilizing purified components of rabbit liver microsomes, it was concluded that the reductase could mediate a low rate of ·OH-dependent oxidation of ethanol, whereas the increase in ethanol oxidation produced by the addition of cytochrome P-450 isozyme 3a

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‖ Abbreviations: ·OH, hydroxyl radical; and KMBA, 2-keto-4-thiomethylbutyric acid.

reflected direct oxidation by the P-450 [10, 11]. On the other hand, the rate of ethanol oxidation has been ascribed entirely to an 'OH-dependent mechanism with cytochrome P-450 required to produce 'OH, presumably P-450 serving as the iron catalyst [12] or, as more recently proposed, serving to bind chelated iron [13].

The current study was carried out to investigate in more detail the role of 'OH in ethanol oxidation by reconstituted systems, and the role of reductase, P-450-PB and iron in catalyzing the production of 'OH. In our earlier studies [2, 9], the molar ratios of P-450/reductase that were utilized ranged from 0.1 to 0.5, which are considerably lower than the molar ratios found with microsomes (about 10–20). Since cytochrome P-450 increases the rate of production of superoxide and, ultimately, H_2O_2 over the reductase-dependent rates, it was possible that cytochrome P-450 could catalyze production of 'OH above the reductase-dependent rate when the P-450/reductase ratio was increased to higher values than previously employed. It was also of interest to study the influence of various P-450/reductase ratios on production of 'OH and oxidation of ethanol when the concentration of iron-EDTA was varied, since it was possible that rates of superoxide and H_2O_2 production could become limiting when high levels of ferric-EDTA were present in the system. Thus, the addition of cytochrome P-450 could be effective in enhancing production of 'OH in the presence of excess iron-EDTA by increasing the production of superoxide and H_2O_2.

MATERIALS AND METHODS

Cytochrome P-450 and NADPH-cytochrome P-450 reductase were purified from phenobarbital-treated male Sprague–Dawley rats (50–60 g) as described previously [2, 9]. Cytochrome P-450-PB was purified to a specific content of 16 nmoles/mg protein, as described by West *et al.* [14], while the final purification of reductase had a specific activity of 32 units/mg protein. A unit of reductase activity refers to an initial rate of 1 μmole of cytochrome *c* reduced per min at 22° [15].

The oxidation of ethanol or 2-keto-4-thiomethylbutyric acid (KMBA) was assayed as previously described [2, 9]. The reductase system was reconstituted with dilauroyl phosphatidylcholine at a ratio of 0.2 μM reductase: 20 μg phospholipid to form the reductase system, or the reductase system was incubated with various amounts of cytochrome P-450-PB to form the complete reconstituted system. Typical incubations were carried out at 37° in test tubes containing 100 mM potassium phosphate buffer, pH 7.4, reductase alone, or reductase plus P-450 at various ratios, and either 30 mM KMBA or 50 mM ethanol as substrate in a final volume of 1.0 ml. Reactions were initiated by the addition of NADPH (final concentration of 10 mM) and terminated by the addition of HCl (final concentration of 0.25 N). The production of ethylene from KMBA or of acetaldehyde from ethanol was assayed by head space gas chromatography [2, 9]. All values were corrected for zero-time controls which contained HCl added before the NADPH.

The generation of 'OH by the model xanthine oxidase system was carried out at 37° in 25-ml Erlenmeyer flasks containing 100 mM phosphate, pH 7.4, 1 mM hypoxanthine and 10 mM KMBA as substrate in a final volume of 3.0 ml. Reactions were initiated by the addition of 0.1 unit of xanthine oxidase (Sigma Chemical Co., EDTA-free) and terminated with HCl; assays were conducted as described above. In some experiments, cytochrome P-450-PB was reconstituted in phospholipid along with xanthine oxidase to resemble the reductase system.

All reagents, enzymes, resins and chemicals were of the highest grade available. Phosphate buffers were passed through Chelex-100 resins (freshly prepared) to remove metals. All solutions were prepared with Chelex-100-treated water.

RESULTS AND DISCUSSION

Oxidation of KMBA. The data presented in Table 1 corroborate previous results that the reductase alone, in the absence of cytochrome P-450, was sufficient to promote the oxidation of KMBA. Cytochrome P-450 (phenobarbital-induced) was added in various amounts (reductase held constant at 0.20 μM) to produce P-450/reductase ratios of 0.5, 1.25, 2.5 and 5. Varying the P-450/reductase ratio over a 10-fold range resulted essentially in no major effect on the oxidation of KMBA. Although small, there was a consistent trend towards a slight stimulation of KMBA oxidation at P-450/reductase ratios of 0.5 to 1.25, whereas a slight decrease was observed

Table 1. Effect of cytochrome P-450 on the oxidation of KMBA by NADPH–cytochrome P-450 reductase

Concentration of P-450 (μM)	Ratio P-450/Reductase	Oxidation of KMBA (nmoles ethylene) 15 min	30 min
0		0.22 ± 0.05	0.61 ± 0.09
0.1	0.5	0.28 ± 0.08	0.71 ± 0.10
0.25	1.25	0.32 ± 0.18	0.72 ± 0.18
0.50	2.5	0.23 ± 0.05	0.69 ± 0.15
1.0	5.0	0.18 ± 0.02	0.52 ± 0.02

The oxidation of KMBA by the reductase (0.2 μM final concentration) in the absence and presence of the indicated concentrations of cytochrome P-450 was measured as described in Materials and Methods. Results are from three experiments.

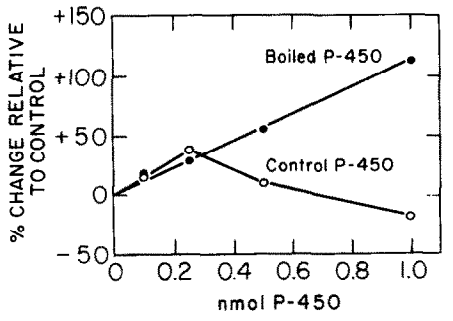

Fig. 1. Effect of native and boiled cytochrome P-450 on the oxidation of KMBA by NADPH–cytochrome P-450 reductase. Reaction conditions are described in the legend to Table 1. Reductase was held constant at 0.2 μM. The control rate of ethylene production was 0.22 nmole for a 15-min reaction period. Results are from two experiments, in duplicate.

at the highest ratio tested, 5 (Table 1). However, the data in Fig. 1 show that the small increase in KMBA oxidation produced by lower concentrations of P-450-PB appeared to reflect a non-enzymatic reaction as evidenced by the fact that boiled cytochrome P-450-PB produced an essentially identical stimulation as control P-450 when added to the reductase system. These results suggest the possibility that the small stimulation by added P-450-PB reflects the presence of non-enzymatic components in the preparation, e.g. iron or EDTA or both (see below).

In the presence of 0.1 mM EDTA, the reductase-dependent oxidation of KMBA was enhanced 3-fold (Table 2; reductase-dependent rate of 1.9 ± 0.4 as compared to the 30-min control value of 0.61 ± 0.09 shown in Table 1). This increase probably reflects the chelation of small amounts of adventitious iron still present in the reaction system even after Chelex treatment [2, 9]. The addition of 15 and 50 μM ferric-EDTA (1:2 iron: EDTA chelate) resulted in increases of ethylene production over the basal levels of 90- and 240-fold respectively (Table 2). Based on those values, it can be calculated that the presence of about 0.5 to 1 μM ferric-EDTA is sufficient to produce the rates observed in the absence of added iron-EDTA.

The addition of cytochrome P-450-PB to produce P-450/reductase ratios of 0.5 to 5 did not result in any increase in the oxidation of KMBA in the EDTA system or the ferric-EDTA systems (Table 2). In fact, as the P-450/reductase ratio was elevated, inhibition of KMBA oxidation was observed under all conditions, with the greatest extent of inhibition occurring at the more physiologically-relevant P-450/reductase ratio of 5 (Table 2). Comparing results of Tables 1 and 2, it is apparent that the inhibitory effect of added cytochrome P-450-PB on KMBA oxidation is increasingly manifested under conditions in which ·OH production is increased, e.g. addition of either EDTA or ferric-EDTA, suggesting that the P-450 does not promote the production of ·OH.

To gain further insight into the possible role that iron may play in these reaction systems, the effect of the potent iron chelating agent, desferrioxamine, was evaluated. Desferrioxamine chelates iron in such a manner that the iron is ineffective in catalyzing production of ·OH [16, 17]. Table 3 shows that, in the absence or presence of EDTA, desferrioxamine produced about 70% inhibition of KMBA oxidation. Moreover, the slight increase in KMBA oxidation produced by the addition of 0.1 or 0.25 μM cytochrome P-450-PB (to produce P-450/reductase ratios of 0.5 or 1.25, respectively) was sensitive to inhibition by desferrioxamine. Thus, in the presence of desferrioxamine, cytochrome P-450-PB did not stimulate the control rate of KMBA oxidation or the rate in the presence of EDTA. The stimulation of KMBA oxidation by added ferric-EDTA was also sensitive to desferrioxamine; however, the inhibition was inversely related to the amount of ferric-EDTA in the system (Table 3), which probably reflects the competition of the two ligands (EDTA and desferrioxamine) for iron coordination sites. Iron binding constants with desferrioxamine and EDTA are $10^{30.7}$ and 10^{25} respectively [17]. Even in the presence of high levels of ferric-EDTA, the addition of cytochrome P-450-PB in the absence or presence of desferrioxamine did not promote the oxidation of KMBA.

Taken as a whole, the above results suggest that production of ·OH requires iron and is mediated by the reductase, not cytochrome P-450. Apparently, sufficient superoxide and H_2O_2 can result from autox-

Table 2. Effect of cytochrome P-450 on the oxidation of KMBA in the presence of EDTA and ferric-EDTA

Concentration of P-450 (μM)	Ratio P-450/Reductase	Oxidation of KMBA and Effect of P-450					
		EDTA		15 μM Fe^{3+}-EDTA		50 μM Fe^{3+}-EDTA	
		Rate	Effect (%)	Rate	Effect (%)	Rate	Effect (%)
0		1.9 ± 0.4		55.6 ± 4.4		146	
0.10	0.5	2.4 ± 0.3	+26	53.5 ± 6.9	−4	162	+11
0.25	1.25	2.2 ± 0.3	+16	43.2 ± 6.3	−22	127	−13
0.50	2.50	1.6 ± 0.2	−16	32.7 ± 8.4	−41	103	−29
1.0	5.0	1.1 ± 0.1	−42	20.0 ± 2.9	−64	77	−47

The oxidation of KMBA by the reductase (final concentration of 0.2 μM) in the absence and presence of P-450 was measured as described in Materials and Methods. Three reaction systems were utilized; 100 μM EDTA (no added iron); 15 μM ferric-30 μM EDTA; and 50 μM ferric-100 μM EDTA (1:2 ferric–EDTA chelate). Results are from three experiments for the EDTA and 15 μM ferric–EDTA systems, and from two experiments for the 50 μM ferric–EDTA system. Rate refers to nmoles ethylene produced for a 30-min reaction period.

Table 3. Effect of desferrioxamine on the oxidation of KMBA in the absence and presence of cytochrome P-450

Concentration of P-450 (μM)	Concentration of desferrioxamine (μM)	Oxidation of KMBA and effect of desferrioxamine							
		Control		EDTA		15 μM Fe^{3+}-EDTA		50 μM Fe^{3+}-EDTA	
		Rate	Effect (%)	Rate	Effect (%)	Rate	Effect (%)	Rate	Effect (%)
0	0	0.61 ± 0.09		1.87 ± 0.41		55.6 ± 4.4		146	
	500	0.20	−67	0.56	−70	29.4	−47	121	−17
0.1	0	0.71 ± 0.10		2.36 ± 0.32		53.5 ± 6.9		162	
	500	0.23	−68	0.57	−76	29.3	−45	117	−28
0.25	0	0.72 ± 0.10		2.15 ± 0.30		43.2 ± 6.3		127	
	500	0.27	−66	0.67	−69	26.9	−38	107	−16

The oxidation of KMBA by the reductase (0.2 μM final concentration) was assayed in the absence or presence of 0.1 or 0.25 μM cytochrome P-450. Four reaction systems were utilized: control, with no added iron or EDTA; 100 μM EDTA (no added iron); 15 μM ferric-30 μM EDTA; and 50 μM ferric-100 μM EDTA. Results are from two to three experiments. Rate refers to nmoles ethylene produced for a 30-min reaction period. Effect of desferrioxamine varied less than 5% for the two experiments.

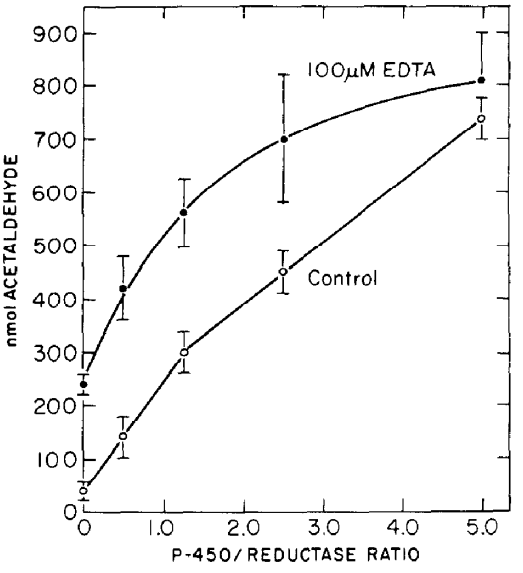

Fig. 2. Effect of cytochrome P-450 on the oxidation of ethanol by NADPH–cytochrome P-450 reductase in the absence and presence of 0.1 mM EDTA. The oxidation of ethanol was assayed as described in Materials and Methods. Results are the mean ± S.E.M. from three experiments. Reductase was held constant at 0.4 μM. A time period of 30 min was utilized.

idation of the reductase or ferrous-EDTA so that the levels of these agents do not reflect a rate-limiting step in the production of ·OH. Hence, the addition of P-450-PB which would generate more O_2^- and H_2O_2 is without effect on the oxidation of KMBA, even in the presence of excess iron. The results also suggest caution in interpreting the effects of added cytochrome P-450 since addition of P-450 could also result in the concomitant addition of small amounts of desferrioxamine-sensitive components, presumably metals such as iron.

Oxidation of ethanol. Data in Fig. 2 show that, analogous to results with KMBA, the reductase alone catalyzed the oxidation of ethanol to acetaldehyde, and that the addition of EDTA itself resulted in about a 6-fold increase in the rate of ethanol oxidation. However, in contrast to results with KMBA, it was noted that acetaldehyde production from ethanol increased as the molar ratio of P-450/reductase increased, even to the highest ratio tested (Fig. 2). The oxidation of ethanol was found to be linear with respect to cytochrome P-450-PB up to a P-450/reductase ratio of 1.25 to 2.5, at which point the reductase may start to become limiting. The addition of cytochrome P-450-PB also resulted in enhanced ethanol oxidation in the presence of EDTA. However, the increase produced by P-450-PB appeared to wane at elevated ratios of P-450/reductase, so that at high concentrations of cytochrome P-450-PB, rates of ethanol oxidation in the absence and presence of EDTA approached each other (Fig. 2). One interpretation of these results is that, in the absence of (iron)-EDTA, whereby the ·OH-dependent oxidation of ethanol is minimized, ethanol is oxidized directly by cytochrome P-450 as are typical drug substrates. When the ·OH-depen-

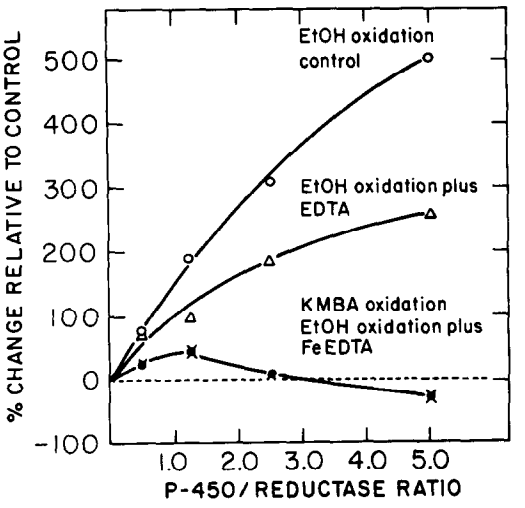

Fig. 3. Effect of cytochrome P-450 on the oxidation of ethanol and KMBA. The oxidation of ethanol was assayed in the absence of any added iron or EDTA (control), or in the presence of 0.1 mM EDTA or in the presence of 50 μM ferric-100 μM EDTA. The control rate of KMBA oxidation was assayed as described in Materials and Methods. Results are from three experiments and are expressed as the percent change affected by the addition of P-450 relative to the rate produced by the reductase alone. Actual rates are similar to those described in Fig. 2 and Tables 1 and 2. Key: (○—○) control ethanol oxidation; (△—△) ethanol oxidation plus 100 μM EDTA; (×—×) ethanol oxidation plus Fe^{3+}-EDTA; and (●—●) control KMBA oxidation.

dent pathway is enhanced such as by the addition of (iron)-EDTA, P-450-PB exerts two opposite effects; stimulation of ethanol oxidation via direct oxidation by P-450 coupled to inhibition of ethanol oxidation via a 'OH-dependent reaction. The latter may be due to scavenging of 'OH or H_2O_2 or binding of iron, or possibly to the fact that tight binding of P-450 to the reductase may prevent or decrease the reductase from transferring electrons to ferric-EDTA.

Support for this interpretation can be seen from the results shown in Fig. 3, which depicts the relative effects of increasing the ratio of P-450/reductase on the rate of oxidation of ethanol or KMBA under conditions of low 'OH generation (controls in the absence of added iron or added EDTA) or in the presence of EDTA, and especially ferric-EDTA, to increase the generation of 'OH. The data are presented as the percent change caused by the increase in the concentration of cytochrome P-450-PB (or increasing P-450/reductase ratio) in order to compare directly the effect of P-450-PB on the various oxidation systems. Such a comparison serves to illustrate the striking difference in the reaction profile between KMBA and ethanol with respect to addition of P-450, when generation of 'OH is limiting. Thus, the oxidation of ethanol increased in consort with the increase in the molar ratio of P-450/reductase, whereas the oxidation of KMBA was only slightly affected. The response of the KMBA system in the presence of EDTA or ferric-EDTA to the varying P-450/reductase ratio was essentially the same as that for the control KMBA system (data not shown in Fig. 3, but essentially that described in Table 2). As the production of 'OH was enhanced by the addition of EDTA, and especially ferric-EDTA, the ethanol oxidation profile tended to resemble the profile which KMBA exhibited (Fig. 3). These results indicate that, unlike a 'OH scavenger such as KMBA, ethanol can be oxidized directly by cytochrome P-450, but the P-450-dependent oxidation of ethanol can be masked under conditions in which the production of 'OH is increased markedly. This may be especially significant if P-450 isozymes which are not particularly effective catalysts of ethanol oxidation, e.g. P-450-PB (LM-2) as compared to 3a type of isozymes, are utilized. Results with ethanol, similar to those with KMBA, show that under conditions of enhanced production of 'OH, cytochrome P-450-PB serves to actually depress the oxidation of ethanol and KMBA. Taken as a whole, it does not appear likely that cytochrome P-450-PB acts as a catalyst for the production of 'OH, but does act as a catalyst for the direct oxidation of ethanol, presumably via a mixed-function oxidase type of reaction [10, 18–20]. This inability of P-450-PB to catalyze the production of 'OH occurs under a variety of P-450/reductase ratios, including ratios approaching those found with intact microsomes, and was observed even under conditions in which excess iron is present in the reaction system. In view of the results of Koop, Coon and colleagues [10, 11, 20], it would also appear that ethanol is

Table 4. Effect of cytochrome P-450 on the oxidation of KMBA by the xanthine oxidase reaction system

Concentration of P-450 (μM)	Oxidation of KMBA and effect of P-450					
	EDTA		15 μM Fe^{3+}-EDTA		50 μM Fe^{3+}-EDTA	
	Rate	Effect (%)	Rate	Effect (%)	Rate	Effect (%)
0	3.4		8.1		12.9	
0.25	2.2	−35	6.1	−25	11.6	−10
0.50	1.8	−47	5.3	−35	9.5	−26
1.0	0.9	−74	3.7	−54	7.0	−46

The oxidation of KMBA by the hypoxanthine-xanthine oxidase system was measured as described in Materials and Methods. Experiments were carried out in the presence of 100 μM EDTA or 15 μM ferric-30 μM EDTA or 50 μM ferric-100 μM EDTA. Results are from two experiments. Rate refers to nmoles ethylene produced per min.

oxidized by P-450 isozyme 3a by a direct oxidation mechanism, rather than by a P-450 catalyzed ·OH mechanism as proposed by others [12, 13].

Studies with xanthine oxidase. Since one mechanism to explain the ability of P-450-PB to depress the oxidation of KMBA and ethanol under conditions of high rates of ·OH production could relate to ·OH-scavenging by the P-450, an additional set of experiments was carried out to study this potential ·OH scavenging ability of cytochrome P-450-PB. The oxidation of hypoxanthine by xanthine oxidase in the presence of ferric-EDTA was employed as an ·OH-generating system [21–23]. As shown in Table 4, the xanthine oxidase system promoted the oxidation of KMBA in a reaction stimulated by the addition of ferric-EDTA. The addition of cytochrome P-450-PB to the system resulted in a decrease in the oxidation of KMBA, analogous to results with the reductase system. There was no inhibition by boiled P-450 (not shown). These results are similar to data observed with the reductase system in which native, but not boiled, P-450 caused a slight decrease in KMBA oxidation (Fig. 1). Studies are in progress to determine if the inhibition by P-450-PB reflects scavenging of ·OH or O_2^- or H_2O_2, or binding of iron (or for microsomal systems, prevention of electron flow to the reductase), but regardless of the exact mechanism, experiments with two different systems suggest that P-450-PB does not act as an effective catalyst for the production of ·OH.

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