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Evidence for Two Ethanol Oxidizing Pathways in Reconstituted Mixed-Function Oxidase Systems[1]

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WINSTON, G. W. AND A. I. CEDERBAUM. *Evidence for two ethanol oxidizing pathways in reconstituted mixed-function oxidase systems.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 189–194, 1983.—The oxidation of ethanol and typical hydroxyl radical scavengers by NADPH-cytochrome P-450 reductase and cytochrome P-450 purified from phenobarbital-treated rats were studied. Ethanol and the scavengers could be oxidized by the reductase system itself. This system was inhibited by superoxide dismutase, competing hydroxyl radical scavengers and desferrioxamine, but stimulated by either EDTA or iron. These results suggest that an iron-catalyzed Haber-Weiss reaction might be involved in the mechanism by which the reductase mediates the oxidation of typical hydroxyl radical scavengers and ethanol. The addition of cytochrome P-450 had no effect on the oxidation of the scavengers, whereas the oxidation of ethanol was enhanced two-to three-fold over the reductase-dependent rate. The oxidation of ethanol was dependent on both the amount of reductase and P-450. There was no effect of competing scavengers, superoxide dismutase or desferrioxamine on the increased rate of ethanol oxidation produced upon addition of cytochrome P-450. Organic hydroperoxides supported the oxidation of ethanol, but not that of the scavengers when added directly to cytochrome P-450. These results suggest that two independent pathways are operative in supporting NADPH-dependent microsomal oxidation of ethanol. One pathway involves hydroxyl radicals which can be generated by the reductase, whereas the other pathway requires the combined presence of both the reductase and cytochrome P-450, and appears to be independent of oxygen radicals.

Ethanol oxidation Reductase system Hydroxyl radical scavengers Mixed-function oxidase systems

TYPICAL hydroxyl radical scavenging agents are oxidized during the NADPH-dependent electron transport by isolated microsomes [1, 6, 7, 15]. These agents include KTBA, methional, Me_2SO, benzoate and several alcohols (l-butanol, isopropanol, t-butanol). Hydrogen peroxide was shown to serve as the precursor of the oxidizing species [1,3]. The rate of oxidation of Me_2SO and KTBA was found to correlate with the specific activity of NADPH-cytochrome P-450 reductase and the specific content of cytochrome P-450 in microsomes isolated from several tissues [16]. Further, KTBA and ethanol were both metabolized by a reconstituted system containing purified components of the microsomal mixed-function oxidase system [5]. These findings suggested that oxygen radical production during microsomal electron transport are at the locus of either cytochrome P-450, NADPH-cytochrome P-450 reductase, or both. The mechanism by which an active oxygen species is produced, resulting in the oxidation of hydroxyl radical scavengers, remains ambiguous.

Recent studies have implicated a role, at least in part, for ·OH, or a species with the oxidizing power of ·OH, in the mechanism whereby microsomes oxidize ethanol. Ethanol oxidation was inhibited by a series of ·OH scavengers in a competitive fashion [1,3]. The addition of iron-EDTA, which increases the production of ·OH by microsomes, increased the oxidation of ethanol [4]. We found previously that ethanol oxidation by cytochrome P-450 purified from phenobarbital-treated rats was inhibited by a series of ·OH scavengers [5]. In that study, it was shown that considerable oxidation of ethanol occurred even in the absence of cytochrome P-450 (25 to 50% of the rate found with the complete system) [5]. The present study represents an attempt to

ABBREVIATIONS

·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical
Me_2SO, dimethylsulfoxide
KTBA, 2-keto-4-thiomethylbutyric acid
SOD, superoxide dismutase

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further elucidate and define the loci at which an active oxygen species may be generated by observing the interaction of several hydroxyl radical scavengers and ethanol with purified, reconstituted components of the rat liver microsomal electron transport system.

METHOD

Methods for the purification of cytochrome P-450 and NADPH-cytochrome P-450 reductase from liver microsomes of phenobarbital-treated rats are described elsewhere [11, 14, 17]. The following reactions were assayed either in the presence of an NADPH-generating system or an organic hydroperoxide: oxidation of ethanol or l-butanol to acetaldehyde or l-butyraldehyde, respectively; production of ethylene from KTBA; production of formaldehyde from Me_2SO, t-butanol or aminopyrine. Assay methods are described elsewhere [1, 6, 7, 15]. Details of individual experiments are presented in legends to tables.

RESULTS

NADPH-Dependent Oxidation of Ethanol and ·OH Scavengers

Initial experiments compared the rate of oxidation of ethanol and 3 typical ·OH scavengers as catalyzed by the complete reconstituted hepatic mixed-function oxidase system to the rate that occurs in the absence of cytochrome P-450 (reductase-dependent system). The production of ethylene from KTBA and the oxidation of either Me_2SO or t-butanol to formaldehyde has been utilized to detect the generation of ·OH by a variety of systems, including the microsomes [1, 6, 7]. Table 1 shows that, in the presence of NADPH and NADPH-cytochrome P-450 reductase, there was a time-dependent oxidation of these 3 ·OH scavenging agents. The addition of cytochrome P-450 to the reaction system virtually had no effect on the oxidation of these ·OH scavengers (Table 1). The oxidation of the scavengers was completely dependent upon the presence of both NADPH and the reductase. The role of phospholipid in the assay appeared to be stimulatory, as indicated by a loss of approximately 20% of the activity in its absence. The production of formaldehyde from 10 mM aminopyrine, a typical mixed-function oxidase substrate, was completely dependent upon the presence of cytochrome P-450.

The rate of ethanol oxidation, either in the absence or presence of cytochrome P-450, was linear over the time course of 30 min (Table 1). While significant oxidation of ethanol occurred in the reductase-dependent system, the addition of cytochrome P-450 resulted in approximately a two- to three-fold increase in the rate of ethanol oxidation (Table 1).

Thus, the reductase alone can account for about 30 to 50% of the ethanol oxidized in reconstituted systems containing components purified from phenobarbital-treated rats. Hence, unique differences exist between the oxidation of ethanol (which appears to involve both a reductase-dependent and a cytochrome P-450 dependent component), typical ·OH scavengers (reductase-dependent only) and classical drug substrates (cytochrome P-450-dependent).

To verify further that the reductase, and not cytochrome P-450, was playing the major role in the ability of the complete reconstituted system to oxidize the ·OH scavengers, titration experiments were performed in which the oxidation of KTBA and t-butanol were measured as a function of varying the concentration of one component (reductase or cytochrome P-450) at a fixed concentration of the other. As shown in Table 2, the rate of KTBA and t-butanol oxidation was dependent solely upon the addition of reductase to the assay mixture. Me_2SO oxidation also showed a direct dependence upon reductase but not cytochrome P-450 concentration (data not shown). The results with ethanol, where the addition of cytochrome P-450 increased the rate of ethanol oxidation over the rate found in the presence of the reductase alone, suggested that ethanol oxidation might be occurring via two independent pathways. If this was in fact the case, then it would be possible to have two rate-limiting components mediating a single event, i.e., ethanol oxidation. As shown in Table 2, ethanol oxidation increased as a linear function of both cytochrome P-450 and the reductase.

TABLE 1

TIME COURSE FOR THE OXIDATION OF KTBA, Me_2SO, t-BUTANOL AND ETHANOL BY PURIFIED COMPONENTS OF THE LIVER MICROSOMAL MIXED-FUNCTION OXIDASE SYSTEM

Substrate	Reaction Time (min)	Activity (nmol product) Reductase	Reductase + P-450
KTBA	2.5	2.4	1.8
	5	4.0	3.8
	10	4.8	5.6
	20	7.0	7.8
Me_2SO	2.5	4.8	5.0
	5	9.6	10.4
	10	19.2	20.4
	20	33.0	35.0
t-Butanol	5	10.0	9.8
	10	18.0	17.0
	15	24.2	25.8
	20	32.4	34.6
Ethanol	5	12.4	32.4
	10	22.9	66.7
	20	57.1	137.1
	30	95.2	226.4

The reaction system contained 100 μg of phospholipid + 10,000 units of reductase in the absence or presence of 1 nmol of cytochrome P-450. The production of ethylene from 10 mM KTBA, or of formaldehyde from either 30 mM Me_2SO or 32 mM t-butanol, or of acetaldehyde from 53 mM ethanol was assayed as described in the Method section.

Effect of Hydroxyl Radical Scavengers, Superoxide Dismutase and Catalase on Oxidation of Ethanol and ·OH Scavengers

Because the reductase alone was shown to catalyze the oxidation of ·OH scavengers, the reductase-dependent pathway of ethanol oxidation probably reflects the interaction of ethanol with ·OH generated from the reductase during NADPH-dependent electron transfer. When cytochrome P-450 was present in the system, there was no enhancement of the reductase-dependent oxidation of the ·OH scavengers, suggesting that the cytochrome P-450-dependent pathway of ethanol oxidation may not involve oxygen radicals. The effect of competitive ·OH scavengers on the rate of ethanol

TABLE 2

OXIDATION OF t-BUTANOL, KTBA AND ETHANOL AS A FUNCTION OF NADPH-CYTOCHROME P-450 REDUCTASE AND CYTOCHROME P-450 CONCENTRATION

Scavenger	Units of Reductase $\times 10^{-3}$	nmol of Cytochrome P-450	nmol of Product per 30 min
KTBA	0	0.5	0
	1.3	0.5	1.0
	2.5	0.5	3.8
	5.0	0.5	6.5
	10.0	0.5	14.0
	10.0	0	20.0
	10.0	0.1	16.5
	10.0	0.3	21.0
	10.0	0.5	18.5
	10.0	1.0	18.0
t-Butanol	0	0.3	0
	1.3	0.3	3.0
	2.5	0.3	8.5
	5.0	0.3	20.5
	10.0	0.3	42.5
	2.5	0	12.0
	2.5	0.1	10.5
	2.5	0.3	9.5
	2.5	0.5	12.0
	2.5	1.0	12.0
Ethanol	0	0.3	0
	1.3	0.3	8.1
	2.5	0.3	17.6
	5.0	0.3	33.8
	10.0	0.3	62.9
	2.5	0	6.2
	2.5	0.1	8.1
	2.5	0.3	14.3
	2.5	0.5	20.5
	2.5	1.0	37.6

Substrate concentrations were KTBA, 10 mM; t-butanol, 32 mM; ethanol, 53 mM. In all experiments, the concentration of phospholipid was held constant at 100 μg per flask.

oxidation catalyzed by the reductase, and by the complete reconstituted system was, therefore, evaluated. Table 3 shows that ethanol oxidation by the reductase-dependent pathway was inhibited by Me_2SO and benzoate. In a similar manner, ethylene production from KTBA was also inhibited by competing ·OH scavengers, including ethanol (Table 3). Thus, ethanol inhibits the oxidation of a ·OH scavenger whereas ·OH scavengers block the oxidation of ethanol. Clearly, a role for ·OH in the reductase-dependent pathway of ethanol oxidation can be discerned. By contrast, when cytochrome P-450 was added to the incubation mixture (note the increase in rate of ethanol oxidation), the ability of Me_2SO and benzoate to act as inhibitors of ethanol oxidation was attenuated (Table 3). In fact, most of the decrease in ethanol oxidation produced by Me_2SO and benzoate in the complete system appears to be due to inhibition of the reductase-dependent activity.

TABLE 3

EFFECT OF COMPETING SCAVENGERS, SUPEROXIDE DISMUTASE AND CATALASE ON THE OXIDATION OF ETHANOL AND KTBA

	Reaction System	Addition	Activity (nmol product/min)	Effect of Addition (%)
A.	Ethanol + Reductase	None	2.7	—
		30 mM Me_2SO	0.9	−66
		30 mM Benzoate	1.5	−46
		0.1 mg SOD	1.4	−48
		0.1 mg Catalase	9.2	+240
B.	KTBA + Reductase	None	0.30	—
		30 mM Me_2SO	0.08	−73
		30 mM Benzoate	0.14	−53
		50 mM Ethanol	0.10	−67
		0.1 mg SOD	0.16	−47
		0.1 mg Catalase	0.08	−70
C.	Ethanol + Reductase Plus P-450	None	6.5	—
		30 mM Me_2SO	5.4	−17
		30 mM Benzoate	5.2	−20
		0.1 mg SOD	4.9	−25
		0.1 mg Catalase	12.3	+90

Substrate concentrations were either 53 (ethanol) or 10 mM (KTBA). The reaction system contained 5,000 units reductase plus 100 μg phospholipid for all experiments. In Experiment C, 0.5 nmol of cytochrome P-450 was also present.

The initial event in the sequelae leading to the production of ·OH is believed to be formation of the superoxide anion radical ($O_2^{\cdot-}$). Dismutation of $O_2^{\cdot-}$ produces H_2O_2, which is probably the precursor of ·OH in biological systems, either via a Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow {\cdot}OH + OH^- + Fe^{3+}$) or an iron-catalyzed Haber-Weiss reaction

$$(O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe} {\cdot}OH + OH^- + O_2)$$

It follows that removal of $O_2^{\cdot-}$ by superoxide dismutase, or removal of H_2O_2 by catalase, should decrease the generation of ·OH and, hence, decrease the oxidation of substrates dependent on interacting with the generated ·OH. As can be seen from the data in Table 3, the oxidation of KTBA was sensitive to both superoxide dismutase and catalase. Thus, there is an apparent role for both $O_2^{\cdot-}$ and H_2O_2 in the reductase-dependent oxidation of KTBA.

Superoxide dismutase inhibited the reductase-dependent oxidation of ethanol by 48%, whereas ethanol oxidation by the completed system was inhibited only 25%. As with the competitive ·OH scavengers, the inhibition of the complete system by superoxide dismutase appears to reflect the inhibition of the reductase-dependent component. Thus, the cytochrome P-450 potentiated activity was essentially insensitive to superoxide dismutase (and ·OH scavengers). Catalase produced a significant increase in the rates of ethanol oxidation in the absence and presence of cytochrome P-450 (Table 3). This probably reflects the peroxidatic activity of catalase-H_2O_2 with ethanol to produce acetaldehyde [13]. The increase in ethanol oxidation produced by catalase indicates that H_2O_2 was being produced by both the reductase, and the complete reconstituted system. In microsomal studies catalase inhibited the oxidation of l-butanol, an alco-

TABLE 4

EFFECT OF IRON-CHELATORS AND IRON ON THE NADPH-CYTOCHROME P-450 REDUCTASE-DEPENDENT OXIDATION OF ETHANOL AND ·OH SCAVENGERS

Substrate	Addition	Activity (nmol product/min)	Effect of Addition (%)
Ethanol	None	1.8	—
	Desferrioxamine	0.7	−61
	EDTA	2.7	+50
	Fe-EDTA	11.4	+530
KTBA	None	0.14	—
	Desferrioxamine	0.06	−57
	EDTA	0.36	+257
	Fe-EDTA	3.70	+2543
Me_2SO	None	0.58	—
	Desferrioxamine	0.23	−60
	EDTA	0.90	+155
	Fe-EDTA	5.30	+814
t-Butanol	None	0.27	—
	Desferrioxamine	0.10	−63
	EDTA	0.45	+166
	Fe-EDTA	4.30	+1493

Substrate concentrations were ethanol, 53 mM; KTBA, 10 mM; Me_2SO, 30 mM; t-butanol, 32 mM. Final concentrations of desferrioxamine, EDTA and Fe-EDTA were 330, 50 and 10 μM, respectively. Reactions were carried out in the presence of 2,500 units of the reductase and 50 μg of dilauroyl phosphatidyl choline. Results are from two or three experiments.

hol that does not serve as an effective substrate for the peroxidatic activity of catalase.

Effect of Desferrioxamine, EDTA and Iron-EDTA

Since reduced iron is necessary for the oxidative decomposition of hydrogen peroxide resulting in hydroxyl radical production, it can be anticipated that iron played a role in the generation of ·OH during NADPH oxidation by the reductase. To determine that iron catalyzed the production of ·OH, the differential properties of two iron-chelators, namely EDTA and desferrioxamine, were exploited. The former is known to form an iron-chelate that potentiates the oxidation of ·OH scavengers, while the latter inhibits the iron-catalyzed production of ·OH in microsomal systems [2,4]. Therefore, a stimulation by EDTA with a concomitant inhibition by desferrioxamine of substrate oxidation would be consistent with a role for iron in generating ·OH in these systems. Table 4 shows that the reductase-dependent oxidation of ethanol, KTBA, Me_2SO and t-butanol was stimulated 50, 257, 155 and 166%, respectively, by 50 μM EDTA, whereas desferrioxamine inhibited the same reactions by approximately 60% in parallel experiments. In view of the above, the addition of iron would be expected to increase the generation of ·OH, and subsequently augment the oxidation of ·OH scavengers and ethanol. The addition of 10 μM iron-EDTA resulted in a large increase in the oxidation of all four substrates (Table 4).

In contrast to the strong inhibition by desferrioxamine of ethanol oxidation by the reductase system, ethanol oxidation by the complete reconstituted system was insensitive to desferrioxamine. The effect of desferrioxamine on ethanol oxidation was 0, −2, and −13 percent in 3 separate experiments.

TABLE 5

OXIDATION OF SUBSTRATES BY CYTOCHROME P-450 IN THE PRESENCE OF ORGANIC HYDROPEROXIDES

Substrate	Product	Activity t-Butyl-OOH (nmol/min/nmol P-450)	Activity Cumene-OOH (nmol/min/nmol P-450)
10 mM Aminopyrine	Formaldehyde	4.38	5.05
53 mM Ethanol	Acetaldehyde	1.43	0.91
74 mM l-Butanol	Butyraldehyde	0.68	1.63
30 mM KTBA	Ethylene	0	0
36 mM t-Butanol	Formaldehyde	0	0

Reactions were carried out in 100 mM potassium phosphate, pH 7.4, containing 0.5 nmol cytochrome P-450 and 100 μg of phospholipid. No reductase or NADPH was added. Reactions were initiated by the addition of either 0.6 mM cumene hydroperoxide or 1 mM t-butyl hydroperoxide.

Organic Hydroperoxide Supported Reaction Systems

Organic hydroperoxides can support the oxidation of drugs and of ethanol by cytochrome P-450, in the absence of NADPH, reductase and molecular oxygen [10]. This type of activity reflects the ability of cytochrome P-450 to act as a peroxygenase. Table 5 shows that both cumene- and t-butyl hydroperoxide promoted cytochrome P-450-dependent oxidation of ethanol and l-butanol in a manner analogous to the oxidation of aminopyrine. By contrast, KTBA, a classic ·OH scavenger, was not a substrate for this peroxygenase activity of cytochrome P-450. Moreover, whereas primary alcohols such as ethanol and l-butanol were oxidized by this system, a tertiary alcohol, t-butanol, was not (Table 5).

The above results suggest that the oxidation of ethanol by the organic peroxide-dependent system reflects a cytochrome P-450 mediated reaction only, with little or no role for ·OH. It should, therefore, follow that this peroxygenase supported oxidation of ethanol would be sensitive to inhibitors of the mixed-function oxidase activity but insensitive to competing ·OH scavengers. Indeed, this proved to be the case as the mixed-function oxidase inhibitor, metyrapone, produced 53 and 40% inhibition of the cumene- and t-butyl-hydroperoxide supported oxidation of ethanol, respectively. In contrast, 30 mM Me_2SO did not inhibit this peroxygenase-dependent oxidation of ethanol by cytochrome P-450.

DISCUSSION

The interaction of ethanol and typical hydroxyl radical scavenging agents with purified, reconstituted components of the microsomal mixed-function oxidase system has been investigated. The data presented herein demonstrate that an active oxygen species with the characteristics of a ·OH can be generated by NADPH-cytochrome P-450 reductase, in the absence of cytochrome P-450. The oxidation of the ·OH scavengers is sensitive to inhibition by superoxide dismutase, catalse, competitive ·OH scavengers and by desferrioxamine, but is stimulated by iron-EDTA and by EDTA itself. These results indicate that O_2^-, H_2O_2 and iron play a role in the production of the oxidant by the reductase,

and, further, that an iron-catalyzed Haber-Weiss type of reaction is responsible for its production.

Results described in this report clearly indicate that oxidation of ·OH scavengers can occur in the absence of cytochrome P-450, and that addition of cytochrome P-450 to the incubation system has no effect on the oxidation of the scavengers. This was in agreement with previous results that suggested that the oxidation of ·OH scavengers could be disassociated from the typical mixed-function oxidase activity of cytochrome P-450 [2–4]. The results suggest that the role of cytochrome P-450 in promoting drug oxidations either differs from its role in catalyzing ·OH production, i.e., acting as a catalyst of the Fenton reaction or that cytochrome P-450 plays little or no role in the generation of ·OH. It is recognized that, because we used the phenobarbital-inducible isozyme of cytochrome P-450 in these studies, some caution should be exercised in interpreting these results. For instance, one could envision that the ·OH scavengers might serve as more suitable substrates for other isozymes of cytochrome P-450. Another possibility that cannot be excluded is that, when the cytochrome P-450 is added to the reductase, a shift in the locus of ·OH production occurs, i.e., the cytochrome P-450, by mediating an efficient electron transfer, would serve to reduce the probability of autoxidation of the reductase. Autoxidation of oxy-cytochrome P-450 would then result in the production of oxyradicals. Further studies will be needed to clarify these possibilities.

The results with regard to ethanol oxidation suggest that a duality exists for the mechanism of the NADPH-dependent oxidation of ethanol by microsomes. Similar to the typical ·OH scavengers, ethanol could be oxidized by the reductase alone. This pathway of ethanol oxidation was sensitive to inhibition by competitive ·OH scavengers, superoxide dismutase and desferrioxamine, but was stimulated by either EDTA or by iron. These results suggest that the reductase-dependent pathway of ethanol oxidation can be attributed to the interaction of ethanol with ·OH generated by the reductase via an iron-catalyzed Haber-Weiss reaction.

On the other hand, interaction with ·OH does not appear to be the sole mechanism by which ethanol is oxidized by microsomes or reconstituted systems. Titration curves demonstrate a dependence of ethanol oxidation on both the reductase and on the cytochrome P-450. That the cytochrome P-450 dependent ethanol oxidation system does not appear to involve ·OH can be seen from the weak extent of inhibition produced by competitive ·OH scavengers, superoxide dismutase and desferrioxamine. In fact, the inhibition of ethanol oxidation by all of these agents can be ascribed to inhibition of the reductase-dependent, i.e., the ·OH-dependent oxidation of ethanol. An important finding is that ethanol and l-butanol, but not KTBA and t-butanol, serve as substrates for the organic hydroperoxide-supported peroxygenase activity of cytochrome P-450. The oxidation of ethanol in this system is inhibited by metyrapone, but not by Me_2SO. These results indicate a mechanism of ethanol oxidation which involves an oxygenated cytochrome P-450 intermediate that is independent of ·OH and does not support the oxidation of ·OH scavengers.

The above results suggest that ethanol is oxidized by two primary pathways in isolated microsomes, one which involves interactions of ethanol with ·OH, the other which appears to be independent of oxyradicals but rather involves cytochrome P-450. Since ethanol oxidation by isolated microsomes is inhibited 40 to 60% by desferrioxamine [2] and 35 to 60% by competing ·OH scavengers [3], it appears that the two independent pathways are contributing about equally to the overall metabolism of ethanol. The contribution made by each pathway can be dramatically altered. The ·OH-dependent pathway can be elevated markedly if microsomal iron is increased. Of interest is the fact that alcoholic liver disease is often associated with iron-overload in the liver [9]. The cytochrome P-450-dependent pathway can be enhanced by inducing the alcohol preferring isozyme of cytochrome P-450 by chronic administration of ethanol [8,12].

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