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THE EFFECT OF EDTA AND IRON ON THE OXIDATION OF HYDROXYL RADICAL SCAVENGING AGENTS AND ETHANOL BY RAT LIVER MICROSOMES

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Rat liver microsomes catalyzed an NADPH-dependent oxidation of dimethylsulfoxide, 2-keto-4-thiomethylbutyrate and ethanol. The addition of EDTA and iron (ferric)-EDTA increased the oxidation of the hydroxyl radical scavenging agents and ethanol. Unchelated iron had no effect; therefore, appropriately chelated iron is required to stimulate microsomal production of hydroxyl radicals. Catalase strongly inhibited control rates as well as EDTA or iron-EDTA stimulated rates of hydroxyl radical production whereas superoxide dismutase had no effect. The rate of ethanol oxidation was ten- to twenty-fold greater than the rate of oxidation of hydroxyl radical scavengers in the absence of EDTA or iron-EDTA, suggesting little contribution by hydroxyl radicals in the pathway of ethanol oxidation. In the presence of EDTA or iron-EDTA, the rate of ethanol oxidation increased, and under these conditions, hydroxyl radicals appear to play a more significant role in contributing toward the overall oxidation of ethanol.

Rat liver microsomes can produce a potent oxidant with properties similar to that of the hydroxyl radical (·OH) during NADPH-dependent electron transfer. The microsomes catalyzed the oxidation of a variety of ·OH scavengers by a reaction which was sensitive to inhibition by competitive ·OH scavengers, to catalase, and to the potent iron-chelating agent, desferrioxamine (1,2). The oxidation of ethanol by microsomes was also inhibited, in part, by competitive ·OH scavengers (3) and by desferrioxamine (2) suggesting some role for ·OH in the mechanism of the microsomal pathway of ethanol oxidation. Based upon experiments with desferrioxamine (2), with organic hydroperoxides (4), and with reconstituted systems containing NADPH-cytochrome c reductase and cytochrome P-450 purified from phenobarbital-treated rats (5,6), it has been suggested that the oxidation of ethanol by microsomes involves two pathways. One pathway involves ·OH which can be generated by the reduc-

Abbreviations: ·OH, hydroxyl radical, or a species with the oxidizing power of the hydroxyl radical; DMSO, dimethylsulfoxide; KTBA, 2-keto-4-thiomethylbutyric acid.

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tase, whereas the other pathway requires cytochrome P-450 and appears to be independent of (free) ·OH.

Iron appears to play a critical role in the production of ·OH by most biological systems, either by a Fenton-type reaction (7) or by an iron-catalyzed Haber-Weiss type of reaction (8,9). EDTA-chelated iron appears to be especially effective in catalyzing the production of ·OH (10,11). The addition of iron-EDTA to microsomes was shown to result in the production of a characteristic spin-trapped adduct between ·OH and the free radical, spin-trapping agent, 5,5-dimethyl-1-pyroline-n-oxide (12,13). To attempt to further distinguish between the two pathways of ethanol oxidation, and to define more clearly the requirement for iron in ·OH production by the microsomal system, the effect of EDTA, and of iron, in an unchelated form as well as in an EDTA-chelated form, on the generation of ·OH and the oxidation of ethanol was studied.

METHODS

Rat liver microsomes were prepared from male Sprague-Dawley rats, washed twice, and resuspended in 125 mM KCl. Protein was determined by the method of Lowry et al. (14). The water and all buffers were routinely passed through a column containing Chelex-100 resin to remove metal contaminants. All reactions were carried out at 37° C in 25 ml Erlenmeyer flasks. The basic reaction mixture consisted of 100 mM potassium phosphate, pH 7.4, 10 mM $MgCl_2$, 0.3 mM $NADP^+$, 1 mM sodium azide (except where indicated otherwise), about 2 mg of microsomal protein and the appropriate substrate in a final volume of 1.0 ml. Three different substrates were utilized: Ethanol, 50 mM; DMSO, 33 mM; and KTBA, 10 mM. The reactions were initiated by the addition of 10 mM glucose 6-phosphate plus 2.3 units of glucose 6-phosphate dehydrogenase, and were terminated after 10 (KTBA and ethanol experiments) or 30 (DMSO) min by the addition of 0.3 ml 1N HCl (KTBA and ethanol) or 0.3 ml of 17% w/v trichloroacetic acid. Zero-time controls contained the acid added before the NADPH-generating system. The production of ethylene from KTBA and acetaldehyde from ethanol was measured by head space gas chromatography as previously described (1). Peak heights were quantified by comparison to standard curves. The production of formaldehyde from DMSO was measured by the method of Nash (15) on 0.9 ml aliquots of the TCA supernatant fraction. All values refer to mean ± SEM.

RESULTS

In the absence of added iron or added EDTA, microsomes catalyzed an NADPH-dependent oxidation of DMSO, KTBA and ethanol (Table I). The rate of ethanol oxidation was an order of magnitude greater than the rate of oxidation of DMSO and KTBA. The addition of ferric-EDTA produced a concentration-dependent increase in the rate of ethanol oxidation, confirming previous results (16). Associated with this ferric-EDTA-dependent increase in ethanol oxidation was an

TABLE I

EFFECT OF IRON, IRON-EDTA AND EDTA ON MICROSOMAL OXIDATION OF KTBA, DMSO AND ETHANOL

Addition	Concentration (mM)	Rate of Substrate Oxidation (nmol/min/mg microsomal protein) KTBA	DMSO	Ethanol
Control	-	0.68 ± 0.04	0.23 ± 0.05	5.75 ± 0.40
Ferric ammonium sulfate	0.005	0.70 ± 0.09	0.22 ± 0.06	5.49 ± 0.08
	0.025	0.73 ± 0.06	0.23 ± 0.07	5.80 ± 0.45
	0.10	0.59 ± 0.04	0.19 ± 0.03	5.05 ± 0.26
Ferric EDTA[a]	0.005	1.01 ± 0.09[c]	0.32 ± 0.07	6.67 ± 0.71
	0.025	2.63 ± 0.34[d]	2.12 ± 0.33[c]	14.31 ± 1.26[d]
	0.10	5.13 ± 0.66[d]	4.44 ± 0.41[d]	24.05 ± 2.21[d]
EDTA	0.01	1.05 ± 0.14[b]	0.48 ± 0.13	6.82 ± 0.22
	0.05	1.64 ± 0.10[d]	1.54 ± 0.09[d]	10.78 ± 0.83[c]
	0.20	1.98 ± 0.11[d]	2.31 ± 0.19[d]	13.29 ± 0.75[d]

[a] Ferric ammonium sulfate dissolved in EDTA in a 1:2 iron-EDTA molar ratio. [b] $p<0.05$. [c] $p<0.01$. [d] $p<0.001$.

increase in the oxidation of KTBA and, especially, DMSO (Table I). Ferric-EDTA had no effect in the absence of NADPH or microsomes or with "zero-time" controls. In contrast to the stimulation produced by ferric EDTA, ferric ammonium sulfate had no effect on the oxidation of ethanol, DMSO or KTBA (Table I).

In view of the stimulation by EDTA-chelated iron, and the lack of effect of iron alone, studies were carried out to determine the effect of EDTA itself on the oxidation of ethanol, DMSO and KTBA. EDTA produced a concentration-dependent increase in the oxidation of the three substrates (Table I). At lower concentrations, the stimulation by EDTA was almost as impressive as the stimulation by ferric-EDTA.

To provide information on the mechanism underlying the stimulation by EDTA and by ferric-EDTA, the effects of superoxide dismutase and catalase on the oxidation of KTBA were studied. Even at a very high concentration (300 units) superoxide dismutase had little or no effect on the control rate of KTBA oxidation, or on the stimulation produced by EDTA or by ferric-EDTA (Table II). The slight inhibition observed at the 200 μM EDTA or 100 μM ferric-200 μM EDTA concentration was also found with boiled superoxide dismutase and therefore appears to reflect a non-specific effect.

Isolated microsomes are usually contaminated with catalase which can remove H_2O_2, a precursor of ·OH. Therefore, all the above experiments were carried out

TABLE II

EFFECT OF SUPEROXIDE DISMUTASE AND CATALASE ON MICROSOMAL OXIDATION OF KTBA

Reaction Condition	Rate of Oxidation of KTBA (nmol ethylene/min/mg microsomal protein)				
	Control	0.05 mM EDTA	0.20 mM EDTA	0.025 mM Fe-EDTA	0.10 mM Fe-EDTA
Control[a]	0.53	1.14	1.56	1.51	4.72
SOD[a,b]	0.53	1.12	1.23	1.50	3.53
Boiled SOD[a,b]	0.55	1.03	1.40	1.77	3.86
Minus Azide	0.38	0.47	0.45	1.08	4.11
Minus Azide + Catalase[c]	0.19	0.07	0.06	0.11	1.13
Minus Azide + Boiled Catalase[c]	0.34	0.52	0.49	0.91	3.99

[a] These flasks all contained 1 mM azide. [b] 300 units of SOD. [c] 65 units of catalase.

in the presence of azide, an inhibitor of catalase. When azide was omitted, and the reaction system supplemented with additional catalase, the control rate of KTBA oxidation was significantly lowered (Table II). Neither EDTA nor the lower concentration of ferric-EDTA was stimulatory in the presence of catalase (Table II). Stimulation was found at the higher concentration of ferric-EDTA, suggesting some effective competition with catalase for H_2O_2. Boiling the catalase for 15-20 min reversed the inhibition and restored rates of KTBA oxidation to the rates found in the absence of azide (Table II). The experiments with catalase suggest that H_2O_2 is the precursor of ·OH, in the absence as well as in the presence of iron-EDTA.

DISCUSSION

It appears that in biological systems, iron is required for the generation of ·OH, either by a Fenton-type of reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$) or by a Haber-Weiss reaction ($Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$; $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$). The production of ·OH during microsomal electron transfer also appears to require iron since the potent iron chelating agent, desferrioxamine, inhibited the oxidation of ·OH scavenging agents by 90% or more (2). Results in the current report show that rat liver microsomes catalyze the oxidation of ·OH scavenging agents and ethanol and that this oxidation is stimulated by the addition of either EDTA itself, or by ferric-EDTA. The stimulation produced by EDTA itself probably reflects chelation of adventitious iron present in the isolated microsomes, since

iron was removed from all solutions by treatment with Chelex resin. However, in contrast to ferric-EDTA, iron alone is not sufficient to stimulate the production of ·OH since ferric ammonium sulfate does not increase the oxidation of DMSO, KTBA or ethanol. Chelated iron has been shown to be necessary for the stimulation of microsomal lipid peroxidation (17,18).

There are several possibilities that may explain the stimulation by chelated iron of the production of ·OH by microsomes. In view of the presence of phosphate buffer, unchelated iron will form ferric phosphate which is very poorly soluble in aqueous, neutral solution. Unchelated iron may be sequestered by microsomal components in such a manner that it is not readily available to participate in the production of ·OH. Since it is iron in the ferrous state which is required to react with H_2O_2 and produce ·OH, unchelated ferric iron may not be reduced to the ferrous form as readily as chelated ferric iron. Chelation of iron is known to change the redox potential of the iron. Reduction of iron by the microsomal system may be mediated by $O_2^{\cdot-}$, which is produced during microsomal electron transfer, or by the flavoprotein, NADPH-cytochrome P-450 reductase. Ferric-EDTA can be directly reduced by the reductase (19,20). The lack of sensitivity to superoxide dismutase suggests that $O_2^{\cdot-}$ might not serve as the primary reducing agent for ferric-EDTA, although it is possible that the superoxide dismutase does not effectively penetrate or have access to the site(s) of $O_2^{\cdot-}$ production. Further experiments are being carried out to investigate these possibilities.

The oxidation of ethanol by rat liver microsomes appears to involve two pathways, one dependent on ·OH, while the other involves a cytochrome P-450, ·OH-independent mechanism (2,4-6). In the absence of EDTA or iron-EDTA, the rate of oxidation of DMSO and KTBA is ten- to twenty-fold lower than the rate of ethanol oxidation (Table I). Therefore, it appears that in the absence of EDTA or iron-EDTA, ethanol is oxidized primarily by the ·OH-independent pathway of rat liver microsomes. However, in the presence of EDTA or iron-EDTA, the rate of oxidation of ethanol, KTBA and DMSO are all increased; under these conditions, ·OH appears to play a more significant role in contributing toward the overall oxidation of ethanol. In fact, desferrioxamine had little effect on the oxidation

TABLE III

EFFECT OF DESFERRIOXAMINE ON MICROSOMAL OXIDATION OF ALCOHOLS

Alcohol	Concentration of Desferrioxamine (mM)	Rate of Alcohol Oxidation -EDTA (nmol/min/mg microsomal protein)	Rate of Alcohol Oxidation +EDTA[b] (nmol/min/mg microsomal protein)
Ethanol[a]	0	7.6 ± 0.4	16.7 ± 2.4
	0.25	6.3 ± 0.8 (-17%)	8.3 ± 0.7 (-50%)
2-Butanol[a]	0	8.5 ± 1.1	13.8 ± 1.3
	0.25	7.6 ± 1.0 (-11%)	8.9 ± 0.8 (-36%)

[a] Concentrations of ethanol and 2-butanol were 50 and 33 mM, respectively.
[b] Added to a final concentration of 0.1 mM.

of alcohols (ethanol and 2-butanol) in the absence of EDTA, but produced inhibition of alcohol oxidation in the presence of EDTA (Table III), confirming a partial role for ·OH in ethanol oxidation when EDTA or iron-EDTA are present. Experiments are in progress to evaluate the ability of other iron-chelates to stimulate the oxidation of ethanol and the production of ·OH by microsomes and by reconstituted systems.

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