A Reexamination of the Cytochrome P-450-catalyzed Free Radical Production from a Dihydropyridine

EVIDENCE OF TRACE TRANSITION METAL CATALYSIS*

(Received for publication, March 22, 1990)

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Radical production from 3,5-bis((ethoxycarbonyl)-4-ethyl-2,6-dimethyl-1,4-dihydropyridine (DDEP) in rat liver microsomes has been attributed to one-electron oxidation via cytochrome P-450 followed by extrusion of an ethyl radical. In the presence of the spin trap \( \alpha-(4\text{-pyridyl 1-oxide})-N\text{-tert-butylnitrone (4-POBN)} \), this radical was detected as the 4-POBN/ethyl radical adduct by electron paramagnetic resonance spectroscopy. The addition of catalase resulted in over a 50% decrease in radical production. The concentration of the 4-POBN/ethyl radical adduct increased about 9-fold in the presence of EDTA and approximately 2-fold in the presence of either diethylenetriaminepentaacetic acid (DTPA), deferoxamine mesylate (DMSO), or deferoxamine mesylate-washed microsomes and NADPH in Chelex-treated incubation buffer, much less 4-POBN/ethyl radical formation occurred. Addition of either DTPA, EDTA, or ferric complexes of these chelators greatly stimulated production of the 4-POBN/ethyl radical adduct in this system. These results suggest that the ethyl radicals produced from DDEP in a microsomal system arise via trace transition metal-catalyzed reactions.

3,5-Bis((ethoxycarbonyl)-4 ethyl 2,6-dimethyl 1,4 dihydro pyridine (DDEP) is a structural analog of the dihydropyridine \( \text{Ca}^{2+} \) antagonists which generally contain a substituted phenyl ring at the 4-position (1). Interest in these analogues developed when it was observed that administration of 3,5-bis((ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine to either mice or rats resulted in elevated levels of protoporphyrin IX and other heme precursors (2-5) via production of the four isomers of \( \text{N} \)-methylprotoporphyrin IX (6) which have been shown to inhibit rat liver ferrochelatase (7). Treatment with 3,5-bis((ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine was also shown to decrease hepatic cytochrome P-450 levels in vivo (8, 9). Production of \( \text{N} \)-alkylprotoporphyrin IX was subsequently shown to arise by transfer of an alkyl group from a 4-alkyl-substituted 1,4-dihydropyridine to the porphyrin (10, 11). DDEP was also shown to cause a time-, NADPH-, and oxygen-dependent loss of cytochrome P-450 in vitro (10). On the basis of these observations, it was argued that heme alkylation is associated with catalytic turnover of 4-alkyl-substituted 1,4-dihydropyridines by cytochrome P-450 (10). Ortiz de Montellano and co-workers (12) provided support for the theory of alkylation by employing the electron paramagnetic resonance (EPR) spin-trapping technique to detect ethyl radicals formed during metabolism of DDEP by hepatic microsomes from rats treated with phenobarbital. They found that production of the \( \alpha-(4\text{-pyridyl 1-oxide})-N\text{-tert-butylnitrone (4-POBN)}/\text{ethyl radical adduct was dependent on the presence of active microsomes, DDEP, NADPH (or NADH), and spin trap (4-POBN) (12). They also found that carbon monoxide inhibited radical production by approximately 65% and suggested, on this basis, that DDEP is oxidized by cytochrome P-450 to a radical cation which subsequently undergoes aromatization via extrusion of an ethyl radical (12). Since this work was done before the possible importance of trace transition metals in microsomal systems was recognized, we have reexamined the mechanism of ethyl radical production from DDEP in a microsomal system to determine whether DDEP is oxidized directly by cytochrome P-450 or whether oxidation occurs by an adventitious transition metal-catalyzed mechanism in which the hydroxyl radical (or an equivalent oxidant) is formed.

MATERIALS AND METHODS

Chemicals—Catalase (EC 1.11.1.6, from bovine liver, thymol-free), superoxide dismutase (EC 1.15.1.1, from bovine erythrocytes), EDTA, diethylenetriaminepentaacetic acid (DTPA), deferoxamine mesylate, NADPH, and D-mannitol were obtained from Sigma. 4-POBN was from Aldrich. Chelex 100 chelating resin was obtained from Bio-Rad. For some experiments, the incubation buffer (0.15 M KCl, 0.1 M sodium phosphate, pH 7.4) was treated with Chelex 100 to remove trace amounts of transition metals by the batch method described by Buettner (13). DDEP was synthesized according to the procedure described by Ortiz de Montellano et al. (10). The m.p. of the purified product was 106-110 °C (literature m.p. 110 °C) (14).

Preparation of Liver Microsomes—Male Sprague-Dawley rats (240-270 g) were administered sodium phenobarbital (80 mg/kg) intraperitoneally for 4 days. Liver microsomes were prepared from these animals 24 h after the final phenobarbital treatment according to the method described by Slater and Sawyer (15) except that the livers were perfused with an ice-cold solution of 0.15 M KCl prior to removal from the rat, and incubation buffer (0.15 M KCl, 0.1 M sodium phosphate, pH 7.4) was used to wash and resuspend the microsomes. A portion of the microsomes was washed in incubation buffer containing 0.5 mM deferoxamine mesylate to remove trace amounts of iron from the preparation. The cytochrome P-450 content of the microsomal suspensions was determined by difference spectroscopy as described by Omura and Sato (16). An Aminco DW-2A UV-visible spectrophotometer was used for the cytochrome P-450 assays.

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RESULTS AND DISCUSSION

A 6-line 4-POBN/radical adduct was observed when liver microsomes from rats treated with phenobarbital were incubated with DDEP in the presence of 4-POBN and NADPH (Fig. 1). The hyperfine coupling constants of this adduct \( \left( a^N = 15.73 \text{ G}, a^0 = 2.62 \text{ G} \right) \) are consistent with those reported by Ortiz de Montellano and co-workers (12) \( \left( a^N = 15.78 \text{ G}, a^0 = 2.73 \text{ G} \right) \) for the 4-POBN/ethyl radical adduct produced by both microsomal metabolism of DDEP and by CuCl\(_2\)-catalyzed decomposition of ethylhydrazine; these workers unambiguously identified this adduct by mass spectrometry. As shown in Fig. 1, the generation of the 4-POBN/ethyl adduct is dependent on the presence of active microsomes, DDEP, NADPH, and 4-POBN. No significant radical production was observed when any of these components was omitted from the incubation system or when microsomes were heat-denatured.

Ortiz de Montellano and co-workers (12) examined the role of reactive oxygen intermediates in the mechanism of ethyl radical formation from DDEP by adding protective enzymes (catalase and superoxide dismutase) and a hydroxyl radical scavenger (mannitol) to the microsomal incubation system. We repeated these experiments with similar results (Table I). The addition of superoxide dismutase had no significant effect on radical production. The addition of catalase resulted in over a 50% decrease in the amplitude of the 4-POBN/ethyl radical adduct. The absence of total inhibition by catalase may indicate that some hydrogen peroxide is formed and reacts in an non-aqueous phase such as the microsomal lipids and is inaccessible to catalase. Since both catalase added after the 30-min incubation period and heat-denatured catalase had no significant effect on radical production, the inhibition observed in the presence of active catalase can be attributed to its ability to remove H\(_2\)O\(_2\) from the incubation. Hydrogen peroxide is produced in microsomes via an NADPH-dependent mechanism (17-19). It has been suggested that H\(_2\)O\(_2\) is formed from superoxide anion which is produced during microsomal electron transport (20). The fact that catalase inhibits its formation of the 4-POBN/ethyl radical adduct suggests that ethyl radical generation from DDEP in a microsomal system is, at least in part, H\(_2\)O\(_2\)-dependent. Oxidation of DDEP by an H\(_2\)O\(_2\)-dependent mechanism may occur via generation of a hydroxyl radical (or its functional equivalent) through a Fenton reaction between ferrous ions and H\(_2\)O\(_2\) (Scheme 1). NADPH-supplemented microsomes have also been shown to reduce Fe\(^{3+}\) to Fe\(^{2+}\) (21). Since the production of both H\(_2\)O\(_2\) and Fe\(^{2+}\) by microsomes is NADPH-dependent, this would explain why production of ethyl radicals from DDEP is also dependent on NADPH.

### Table I

| Additions to incubation system | Relative amplitude |
|-------------------------------|-------------------|
| None (control)                | 100 ± 11          |
| +Catalase (0.1 mg/ml)          | 47 ± 09 (49)      |
| +Superoxide dismutase (0.1 mg/ml) | 88 ± 05 (93)    |
| +Catalase and superoxide dismutase | 56 ± 02 (65)  |
| +Catalase (added postincubation) | 99 ± 06 (100)    |
| +Heat-denatured catalase      | 87 ± 07 (104)     |
| +Mannitol (90 mM)             | 120 ± 08 (105)    |

* The standard incubation contained DDEP (1 mM), microsomes (2.5 nmol/ml cytochrome P-450), EDTA (1.5 mM), 4-POBN (20 mM), and NADPH (1 mM) in 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM KCl.

The low field line of the 4-POBN/ethyl radical adduct signal was measured after a 30-min incubation at 37 °C. The values are the means of three different experiments ± S.E. Values in parentheses are those reported by Ortiz de Montellano and co-workers (12).
Transition Metal-catalyzed Oxidation of a Dihydropyridine

If free hydroxyl radical is the species that oxidizes DDEP to a radical cation, then a hydroxyl radical scavenger would be expected to inhibit this oxidation (and, therefore, subsequent extrusion of an ethyl radical from the radical cation of DDEP). The fact that mannitol does not inhibit radical production, as reported by Ortiz de Montellano and co-workers (12), may be due to the partitioning of this compound into the aqueous phase, that is mannitol may not reach the site of DDEP is localized. This rationale has previously been used to explain the failure of mannitol to protect liposomes from iron-induced lipid peroxidation (22). The apparent increase in 4-POBN/ethyl radical adduct concentration in the presence of mannitol can be attributed to the spin trapping of a mannitol-derived radical which is detected in the absence of DDEP (data not shown); the EPR spectrum of this adduct overlaps with the spectrum of the 4-POBN/ethyl radical adduct.

All previous EPR spin-trapping studies that examined the production of ethyl radicals from DDEP were carried out in un-Chelaxed phosphate buffer in the presence of either EDTA (12) or DTPA (23). Since both isolated microsomes and phosphate buffer contain trace amounts of “free” transition metals, ferric iron-chelator complexes would be expected to exist in microsomal incubations treated with either DTPA or EDTA. Both ferric-EDTA and ferric-DTPA have been shown to be reduced directly by both microsomes and purified cytochrome P-450 reductase to their corresponding ferrous chelates (24, 25). These species can, in turn, react with H2O2 in a Fenton-type reaction to generate the hydroxyl radical (26). Enhancement of hydroxyl radical production in rat liver microsomes by ferric-EDTA and ferric-DTPA has been demonstrated by measuring a stimulation of the rate of oxidation of 2-keto-4-thiomethylbutyric acid, a hydroxyl radical scavenger, in the presence of these iron chelates (25). These results suggest that enhanced hydroxyl radical production does occur in microsomal incubations in the presence of either EDTA or DTPA (Scheme 1).

We examined the effect of metal chelators on the production of ethyl radicals from DDEP in microsomes to determine whether iron chelates are involved in the mechanism of radical production (Fig. 2). Although the 4-POBN/ethyl radical adduct was detectable when microsomes were treated with DDEP and NADPH in the absence of added metal chelator, maximum radical adduct signal intensity was observed when 1.5 mM EDTA was included in the incubation system. Inclusion of either 1.5 mM DTPA or 0.1 mM deferoxamine mesylate resulted in a 4-POBN/ethyl radical adduct concentration approximately one-half that seen in the presence of EDTA. These results suggest that ethyl radical production from DDEP is largely dependent on trace transition metals present in the microsomal system. It is surprising that radical production was observed in the presence of deferoxamine mesylate since ferric-deferoxamine mesylate has generally been viewed as being relatively inert (24, 27, 28). Recently, evidence has been presented that this complex is reduced by microsomes in a reaction dependent on the presence of cytochrome P-450 (21), suggesting that ferric-deferoxamine mesylate is not redox-inert. The fact that the concentration of the 4-POBN/ethyl radical adduct is much greater in the presence of deferoxamine mesylate than in the absence of added che-

Fig. 2. Effect of metal chelators on the kinetic profile for the formation of the 4-POBN/ethyl radical adduct. The trace marked “no chelator” was an incubation containing rat liver microsomes (2.5 nmol/ml cytochrome P-450), 150 mM KCl, 1 mM DDEP, 20 mM 4-POBN, and 1 mM NADPH in 100 mM sodium phosphate buffer (pH 7.4); B, same as in A but with 1.5 mM EDTA added; C, same as in A but with 1.5 mM DTPA added; D, same as in A but with 0.1 mM deferoxamine mesylate added. Other experimental conditions and spectrometer settings are the same as in Fig. 1.
lator suggests that the ferric-deferoxamine mesylate complex is not inert in our system.

To further explore the effect of metal chelators on ethyl radical production from DDEP, we examined the kinetic profile for the formation of the 4-POBN/ethyl radical adduct in the presence and absence of chelators (Fig. 3). This study showed that radical production was more rapid in the presence of EDTA. In the presence of either DTPA or deferoxamine mesylate, radical production occurred at a slower linear rate. When no chelator was added, the initial rate of formation was equal to that seen in the presence of EDTA, although radical production was not as sustained as that seen in the presence of the metal chelators. Thus, the initial rate of 4-POBN/ethyl radical adduct formation in the absence of chelator may also occur via trace iron-catalyzed reactions.

To investigate whether DDEP oxidation and subsequent ethyl radical production is totally dependent on trace transition metal-catalyzed reactions, we examined the kinetic profile of 4-POBN/ethyl radical adduct production in microsomes washed with deferoxamine mesylate to remove trace amounts of “free” iron. The reactions were carried out in incubation buffer pretreated with Chelex 100 resin. Albono et al. (29) have used this approach to show that the α-hydroxyethyl free radical formed via ethanol metabolism in microsomes is produced primarily by a hydroxyl radical-mediated mechanism and that the cytochrome P-450-catalyzed mechanism is only a minor pathway. In the absence of added chelator, there was no significant level of radical production (Fig. 4). The addition of either 11 μM DTPA or 10 μM FeCl3/11 μM DTPA caused only a slight stimulation in the rate. The addition of 11 μM EDTA resulted in a more rapid appearance of the radical adduct. Stimulation of radical production by the chelators alone may reflect chelation of “free” iron still present in the microsomes, or it may be due to the presence of trace amounts of iron in the chelator solution. The addition of either 50 μM FeCl3/55 μM DTPA or 10 μM FeCl3/11 μM EDTA led to an even more rapid rate of radical production. Production of the 4-POBN/ethyl radical adduct observed by Ortiz de Montellano and co-workers (12, 23) appears to have been due to the fact that their microsomal incubations contained reductively-active transition metal (iron?)-chelator complexes.

The results presented herein suggest that oxidation of DDEP to a radical cation (and subsequent extrusion of an ethyl radical) does not occur via a direct reaction between cytochrome P-450 and DDEP. Instead, the data show that production of the 4-POBN/ethyl radical adduct from DDEP in a microsomal system occurs via trace transition metal-catalyzed reactions dependent on the presence of hydrogen peroxide. Since the detection of the 4-POBN/ethyl radical adduct in microsomal incubations is the major EPR evidence for the formation of free radical metabolites by cytochrome P-450 (30), the free radical mechanism for the oxidation of substrates by cytochrome P-450 needs to be reexamined.

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