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PRELIMINARY COMMUNICATIONS

IMMUNOCHEMICAL IDENTIFICATION OF AN IRREVERSIBLY BOUND HEME-DERIVED ADDUCT TO CYTOCHROME P-450 FOLLOWING CCl_4 TREATMENT OF RATS

Helen W. Davies, Hiroko Satoh, Richard D. Schulick and Lance R. Pohl

Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.

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It is well known that the administration of CCl_4 produces substantial decreases in the activities of cytochromes P-450 in the liver, adrenals, and testes (1-4). Although it is clear that the destruction of the heme moiety of cytochrome P-450 is responsible for a significant amount of this decrease of enzymatic activity (1), the mechanism of the heme loss and the products of its degradation have not been elucidated. In the present study, we have found that CCl_4 destroys liver microsomal cytochromes P-450, at least in part, by producing irreversibly bound heme-derived products.

EXPERIMENTAL PROCEDURES

Chemicals. Chemicals were obtained from the following sources: [^{14}C]$NaHCO_3$ (54.0 mCi/mmole) and [3,5-^{3}H]delta-aminolevulinic acid (ALA, 1.8 Ci/mmole) from New England Nuclear (Boston, MA); and DEAE Affi-Gel Blue resin from Bio-Rad (Richmond, CA). Emulgen 911 was a gift from the Kao Corp. (Tokyo, Japan).

Methods. Sprague–Dawley rats (70-100 g, Taconic Farms, Germantown, NY) were pretreated with phenobarbital (80 mg/kg in saline, ip) for 4 days and then administered [^{14}C]$NaHCO_3$ (11.4 mCi/kg, 210 umole/kg) and [3,5-^{3}H]ALA (8.40 mCi/kg, 4.67 umole/kg) to radiolabel microsomal protein and heme, respectively, following essentially the procedure of Parkinson et al. (5). Two hr and 4 hr after the administration of [^{14}C]$NaHCO_3$ and [3,5-^{3}H]ALA, respectively, two rats were injected with either CCl_4 (26 mmole/kg, ip, as a 50% solution in sesame oil) or sesame oil (control rats). After 1 hr, liver microsomes were isolated, resuspended in 10 mM Tris-acetate (pH 7.4) containing 20% (v/v) glycerol and 1 mM EDTA (to give a protein concentration of 20-25 mg/ml) and stored at -80° until used. The activities (dpm/mg protein; average of two values) of the microsomes from the control and CCl_4-treated rats were as follows: control group, [^{3}H]label 280,000, [^{14}C]label 35,000; CCl_4 group, [^{3}H]label 213,000, [^{14}C]label 32,000.

The irreversible binding of [^{3}H]heme-derived radiolabel to microsomal protein was determined by precipitation of microsomes in 5 volumes of

acetone containing 0.5 M HCl. The protein pellets were washed with the acetone-HCl until the counts in the supernatant were background, dissolved in 1.0 N NaOH, and counted by scintillation spectrophotometry.

Radioelectrophoretograms of microsomal proteins were determined by first separating the microsomal proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) essentially by the method of Laemmli (6) in a separating gel (9% acrylamide) 1.5 mm thick and 12 cm long. Secondly, the gels were cut into 2 mm sections, solubilized in 0.5 ml of a 19:1 mixture (v/v) of 30% H_2O_2 and concentrated NH_4OH, and after the addition of 30 ul of glacial acetic, counted by scintillation spectrophotometry.

A major form of cytochrome P-450 (molecular weight 54,000 daltons) (P-450-54 kD) was purified from microsomes of phenobarbital-treated rats by the method of West et al. (7) to a specific activity of 17 nmole/mg protein and apparent homogeneity as determined by SDS-PAGE. Anti-P-450-54 kD serum was raised in female New Zealand White rabbits. The IgG fraction was purified from the serum by DEAE Affi-Gel Blue chromatography as described by the Bio-Rad Laboratories and concentrated by ultrafiltration to a protein concentration of 10 mg/ml.

The immunochemical identification of a P-450-54 kD [^{3}H]heme-derived adduct was determined in the following manner. Microsomes were solubilized to a final concentration of 8 mg/ml in 50 mM potassium phosphate (pH 7.4) containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 M KCl, 1% (w/v) sodium cholate, and 0.2% (v/v) Emulgen 911, and an aliquot (25-30 ug) was mixed with anti-P-450-54 kD IgG to precipitate immunoreactive P-450-54 kD according to the method of Newman et al. (8). After the immunoprecipitates were solubilized in 60 mM Tris-HCl (pH 6.8) containing 2.3% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) mercaptoethanol, samples were analyzed for irreversibly bound [^{3}H]heme-derived label by the acetone-HCl precipitation procedure and radioelectrophoretography as described above. All experiments were repeated with normal IgG; the non-specific binding was subtracted from the above results to give corrected levels of irreversibly bound [^{3}H]heme-derived label.

Cytochromes b_5 and P-450 were determined by the method of Omura and Sato (9) and protein was measured by the method of Lowry et al. (10) with BSA as the standard. All experimental results represent the average of single determinations from two rats.

RESULTS

Carbon tetrachloride caused a 60% loss of spectrally determined cytochromes P-450 one hour after its administration to phenobarbital-treated rats whose microsomal proteins and heme had been prelabeled with [^{14}C] and [^{3}H] respectively. As reported by other investigators, the level of cytochrome b_5 was not affected by CCl_4 treatment (1,3).

Precipitation of microsomal protein with acetone-HCl, which will extract noncovalently bound heme from hemeproteins (11), showed that CCl_4 treatment caused a substantial fraction (28%) of the microsomal [^{3}H]heme-derived label to become irreversibly bound to microsomal proteins. In the

microsomes prepared from control rats, 4% of the [^{3}H]label was irreversibly bound to microsomal protein. Radioelectrophoretograms of the microsomes revealed that the irreversibly bound [^{3}H]label was confined to the fractions which contain 47 kD to 56 kD proteins (results not shown).

Immunoprecipitation of microsomes with anti-P-450-54 kD IgG established definitively that there was heme-derived [^{3}H]label bound irreversibly to a cytochrome P-450 (39% of the irreversibly bound label from the CCl_4-treated rats and 75% of the irreversibly bound label from the control rats). Radioelectrophoretograms of the immunoprecipitates confirmed that the antibody precipitated mainly immunoreactive P-450-54 kD from both control (Fig. 1 A and B) and CCl_4 (Fig. 1 C and D) treated rats.

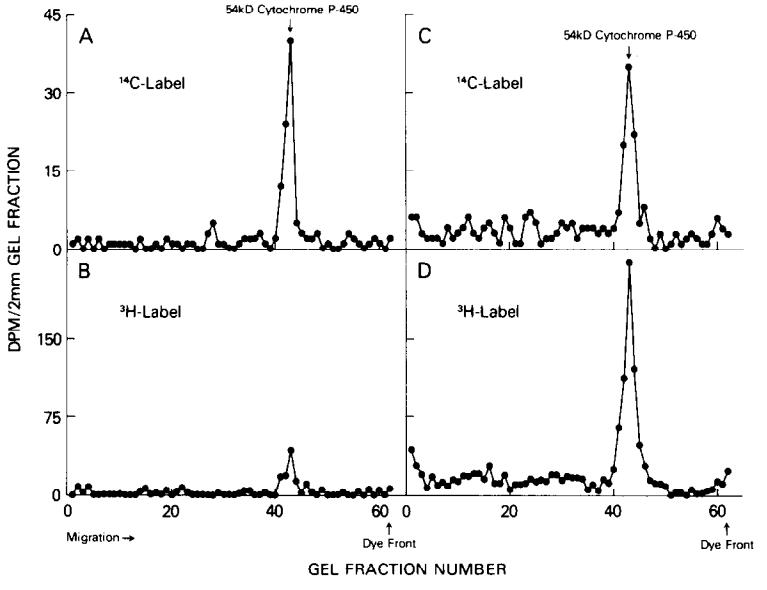

Fig. 1. Radioelectrophoretograms of anti-P-450 54 kD IgG immunoprecipitates of liver microsomes from control (A and B) and CCl_4-treated (C and D) rats.

DISCUSSION

The results of this study show that the destruction of liver cytochromes P-450 produced by CCl_4 *in vivo* is due appreciably to the modification of the heme moiety and the irreversible binding of its degradation product or products to proteins of the endoplasmic reticulum. A substantial portion of the destroyed heme moiety of cytochrome P-450 has been shown unequivocally to be bound to immunochemically reactive P-450-54 kD. Whether or not the remainder of the irreversibly bound heme fragments are also bound to cytochromes P-450 remains to be determined.

Although the mechanism for this destructive process is not clear at the present time, other xenobiotics may destroy cytochromes P-450 by a similar process. In the case of CCl_4, it is possible that one or more of its reactive metabolites such as CCl_3., CCl_3OO., CCl_3OOH, $:CCl_2$, or electrophilic chlorine (12-14) might be directly involved (15-18). Metabolites of CCl_4 might also have an indirect role in the heme

degradation process by promoting the formation of lipid hydroperoxides (13,14,19) which can destroy cytochrome P-450 (20-22). In this regard, organic hydroperoxides have recently been shown to destroy cytochrome P-450 in rat liver microsomes, at least in part, by producing heme degradation products which bind irreversibly to microsomal proteins (23,24). If a similar process occurs slowly in normal animals, it may account for the low level of heme-derived material found irreversibly bound to cytochrome P-450 in normal rats (Fig. 1 A and B).

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