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CYTOCHROME P-450- AND PEROXIDASE-DEPENDENT ACTIVATION OF PROCARBAZINE AND IPRONIAZID IN MAMMALIAN CELLS

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Metabolism of hydrazine derivatives, procarbazine and iproniazid, to reactive free radical intermediates has been studied using spin-trapping techniques in intact human promyelocytic leukemia (HL60) and mouse hepatic cell lines. While HL60 cells have been shown to contain both myeloperoxidase and cytochrome P-450 enzymes, the hepatic cell line shows only cytochrome P-450 activity. Both peroxidases and cytochrome P-450 have been reported to catalyze biotransformation of hydrazines. Procarbazine and iproniazid were rapidly metabolized in these cell lines to methyl and isopropyl radicals, respectively. However, in HL60 cells, procarbazine was metabolized by myeloperoxidase while iproniazid was metabolized mostly by the cytochrome P-450 system. In the hepatic cells, both of these compounds were metabolized by the P-450 system.

KEY WORDS: Procarbazine, iproniazid, P-450-enzymes.

INTRODUCTION

The alkylhydrazine containing drug, procarbazine, N-isopropyl- α-(2-methylhydrazino)-p-toluamide, is active against a number of human cancers including Hodgkin's disease, brain tumors and malignant lymphoma.[1-3] Iproniazid, an antidepressant also contains a hydrazine moiety and has been shown to be extremely toxic to liver.[4] Both procarbazine and iproniazid undergo enzymatic biotransformation to form toxic intermediates.[5-9] Cytochrome P-450 and peroxidases have been implicated in this activation. Thus, cytochrome P-450-dependent metabolism of procarbazine *in vitro* and *in vivo* has been shown to form methylating species which is implicated in its cytotoxicity and other pharmacological properties.

Studies from our laboratory have shown that both cytochrome P-450 and peroxidases (prostaglandin synthase and horseradish peroxidase) catalye the formation of a varietyof free radical species from hydrazine derivatives including procarbazine and iproniazid.[10-15] As free radical species are implicated in cascade of events including toxicity and cell kill, we investigated the formation of these reactive species from procarbazine and iproniazid in intact cells. The purpose of the present work was two-fold: one to directly observe the free radical species in tumor cells and two to gain some insight into the enzymes involved in this transformation. We chose human

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promyelocytic leukemia (HL60) and mouse hepatic (NCTC) cells for these studies. These cells were selected because HL60 cells express both myeloperoxidase and cytochrome P-450 enzymes while hepatic cells only contain cytochrome P-450.

MATERIAL AND METHODS

Procarbazine (NSC 77213) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. Iproniazid phosphate, 3,5-dibromonitrosobenzenesulfonic acid (DBNSB), and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO).

Diethylenetriaminepentaacetic acid (DETAPAC) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

HL60 tumor cells (obtained from Dr. Steve Grant) and mouse hepatic cells (American Tissue Culture, Rockville, MD) were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, and 50 μg/ml gentamycin. For the detection of free radicals, cells were harvested following gentle centrifugation, washed twice with ice-cold phosphate buffered-saline (PBS, pH 7.4) and suspended in PBS at a density of 5×10^6 cells/ml. The reaction mixture for the spin-trapping experiments contained in a final volume of 1 ml: cells (5×10^6) and DBNBS

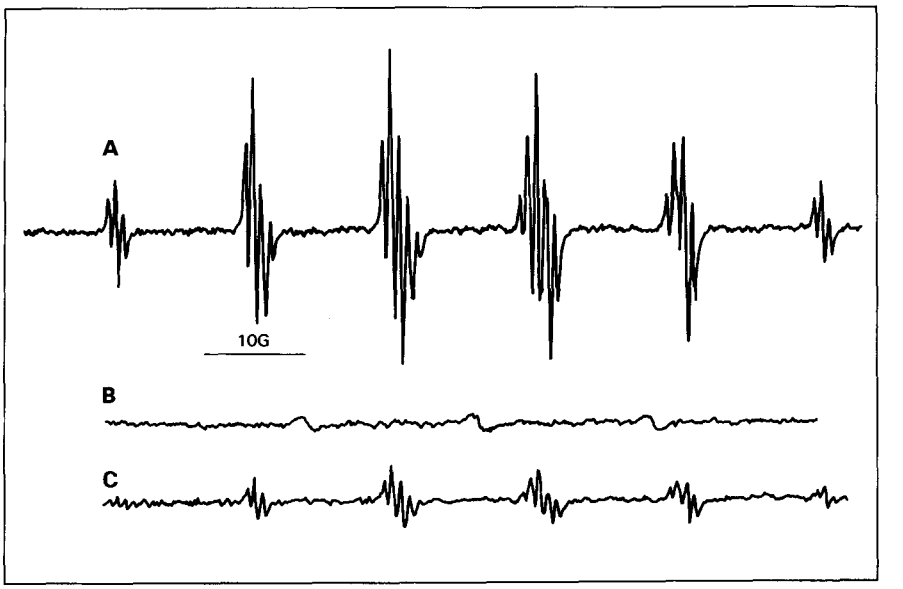

FIGURE 1 The ESR spectrum obtained from 500 μM procarbazine (A) in the presence of DBNBS spin trap (2 mg/ml) with HL60 cells (5×10^6 cells/ml) in PBS; (B) same as (A) except no drug was present; and (C) same as (A) except no cell was present. The ESR settings were: field = 3480 G; microwave power = 20. mW; scan range = 100 G; scan time = 500 sec; modulation ampltude = 1.0 G; time constant = 1.25 sec, and the receiver gain was 2.5×10^5. The ESR parameters for the DBNBS-CH_3 adduct are a^N = 14.5 G; a^3_H = 13.7 G; a^H_m = 0.7 G.

(2 mg/ml). All incubations contained 100 μM DETAPAC to minimize non-enzymatic metal ion-dependent decomposition of the drugs. The reactions were initiated at room temperature by adding the drug (250–500 μM), and recording the resulting spectrum on a ER 220 D IBM-Brucker spectrometer equipped with a TM 110 cavity and operating at 9.5 GHz.

RESULTS

Procarbazine

Incubation of procarbazine with HL60 in the presence of DBNBS resulted in the formation of a well resolved ESR spectrum (Figure 1A) with hyperfine splitting constants of a_N = 14.5 G; a_H^3 = 13.7 G and a_m^H = 0.7 G, resulting from a nitrogen atom, three equivalent methyl protons and two equivalent meta protons. These parameters are consistant with fromation of a spin adduct of methyl radical,[16,17] formed from procarbazine in HL60 cells. In the absence of cells, only traces of this adduct was formed (Figure 1C), indicating enzymatic activation was responsible for the adduct formation. Since peroxidases and cytochrome P-450 can catalyze this formation of methyl radical, we used modulators of cytochrome P-450 system to evaluate the enzyme involved in procarbazine activation in HL60 cells. Incubation of HL60 cells with either SKF-525 A or metyrapone, inhibitors of cytochrome P-450,[13,18] had no significant effects on DBNBS-methyl adduct formation from procarbazine. Addition of H_2O_2, on the other hand, stimualted the adduct formation 2-fold in HL60 cells.

Incubation of procarbazine with the mouse hepatic cells in the presence of the spin trap also resulted in formation of the methyl radical adduct as observed with HL60 cells (Figure 2). However, the relative amounts of the radical formed was lower in this system than with HL60 cells. Pretreatment of hepatic cells with phenobarbital stimulated methyl radical formation in a dose-dependent manner (Figure 2B). However, when the mouse hepatic cells were preincubated with either SKF-525 A or metyropone for 30 min prior to the addition of procarbazine and DBNBS, a 2-3-fold decrease the adduct fromation was observed (Figure 2C).

Iproniazid

Incubation of iproniazid with HL60 cells in the presence of the spin trap also resulted in the formation of an ESR detectable 6-line (Figure 3A) spin adduct spectrum with the following splitting constants: a^N = 14.5 G, a^H = 9.5 G. These parameter are characteristics of an isopropyl radical adduct with DBNBS.[14] Inclusion of either SKF-525 A or metyropone in the incubation mixtures containing iproniazid, and the spin trap decreased the formation of isopropyl radical adduct in HL60 cells (Figure 3B). Addition of H_2O_2 did not effect the radical-aduct formation.

When iproniazid was incubated with the mouse hepatic cells, isoproply radical adduct of DBNBS was also detected with identical ESR hyperfine coupling parameters (Figure 4A). Preincubation of these cells with phenobarbital (100 μM) increased the adduct yield about 2-fold (Figure 4B). In contrast, when SKF-525 A or methyrapone was present in the incubation mixtures, a 2-fold decrease in the free radical formation was observed (Figure 4C).

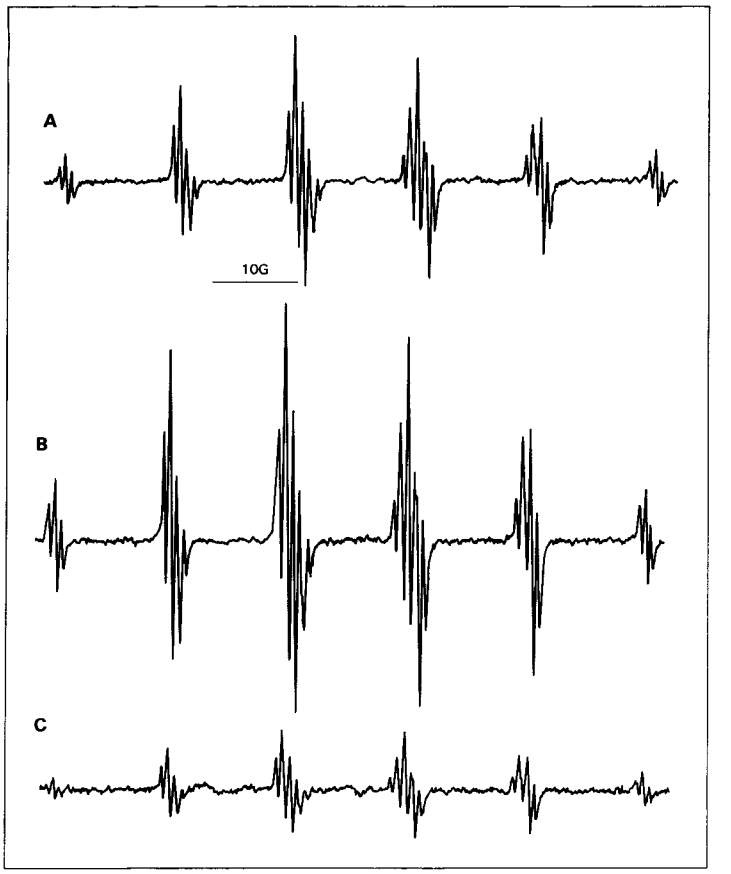

FIGURE 2 The ESR spectrum obtained from 500 μM procarbazine (A) in the presence of DBNBS (2 mg/ml) in mouse hepatic (5×10^6) cells/ml; (B) same as (A) except cells were preincubated with 100 μM phenobarbital for 24 hrs; (C) same as (A) except cells were preincubated with 1 mM metyropone for 30 min on ice. The incubation mixture was then warmed to room temperature before adding the drug and the spin trap. The ESR settings were identical to those described.

DISCUSSION

Hydrazine derivatives represent an important class of drug that are used as an anticancer agent, antidepressant, vasodialator and antituberculosis agent. The pharmacological basis for their action is dependent upon metabolism to reactive species. The cytotoxicity of procarbazine and liver toxicity of isoniazid and iproniazid is reported to involve metabolism to bioalkylating species. Previous studies *in vitro* and *in vivo* from our laboratory have shown that hydrazine and its derivatives form primary and secondary free radical intermediates.[10–15] A number of studies have indicated that these free radicals bind covalently to cellular proteins and DNA for their toxic manifestations.[4,9]

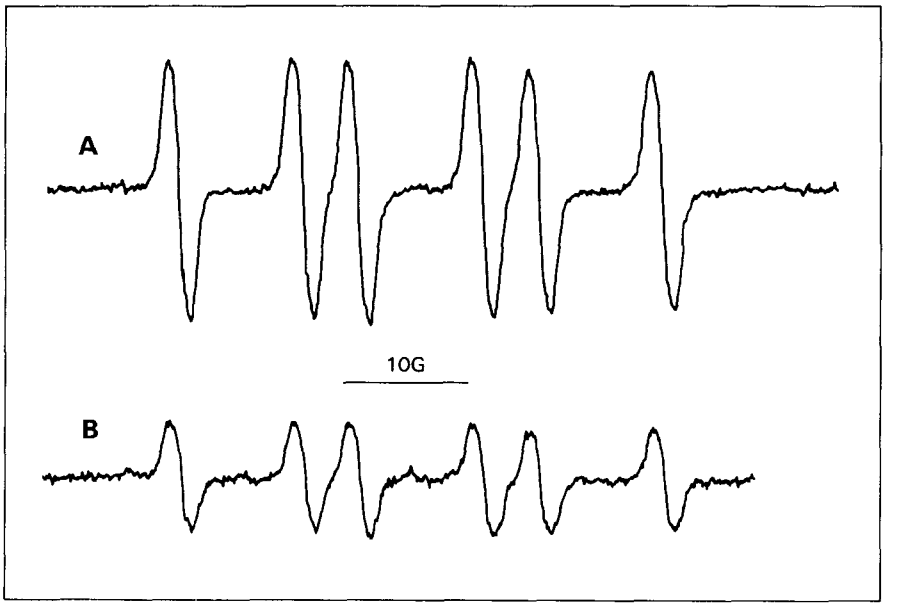

FIGURE 3 ESR spectrum obtained for 1 mM iproniazid (A) in the presence of DBNBS (1 mg/ml) with HL60 (5×10^6) cells/ml; (B) same as (A) except 1 mM SKF-525 A was preincubated with HL60 cells for 30 min on ice before adding the drug and the spin trap. The ESR settings were similar to those described in Figure 1 and the receiver gain was 5×10^5. The ESR parameters for the spin adduct are $a^N = 14.5$ G and $a^H = 9.5$ G.

Studies reported here show that procarbazine and iproniazid form free radical intermediates as a result of enzymatic activation in HL60 and mouse hepatic cells. Methyl radical was the primary radical species formed from procarbazine in both cell lines. However, the mechanism and the activating enzyme for the formation of this radical in these two cell lines appears to be different. For example, the activation of procarbazine in HL60 cells is myeloperoxidase-catalyzed as neither SKF-525 A nor metyraopne, inhibitors of cytochrome P450, affected methyl rdical formation. However, in hepatic cells, cytochrome P-450 appeared to be the enzyme responsible for this metabolism of procarbazine as inhibitors of the P-450 decreased the adduct formation. Furthermore, procarbazine metabolism to methyl radical is catalyzed by phenobarbital inducible monooxygenases.

Iproniazids is metabolized to isopropyl radical as previously detected by peroxidases. However, this study with HL60 cells indicates that peroxidase-catalyzed isopropyl radical formation from iporoniazid is not the major metabolic pathway as addition of H_2O_2 had no effect on radical formation and both metyropone and SKF-525 A inhibited DBNBS adduct formation in HL60 cells. These observations indicate that metabolism of iproniazid is mostly catalyzed by cytochrome P-450. This is further supported by our results with hepatic cells where isopropyl radical formation was inhibted by inhibitors of cytochrome P-450 and induced by phenobarbital.

The present study extends to concept that free radicals are formed from hydrazine

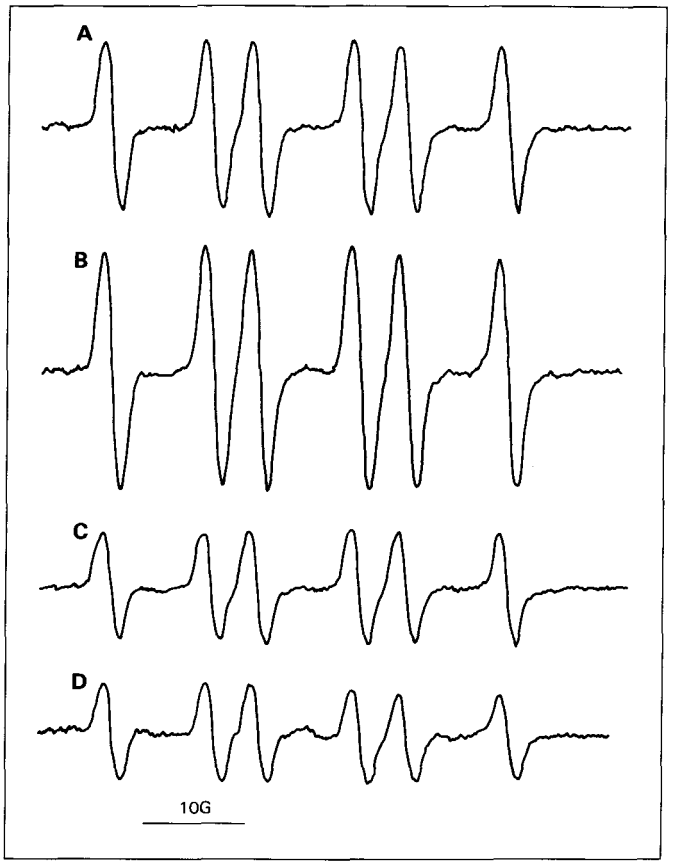

FIGURE 4 ESR spectrum obtained from 1 mM iproniazid (A) in the presence of DBNBS (1 mg/ml) in mouse hepatic (5×10^6 cells/ml); (B) same as (A) except cells were preincubated with 100 μM phenobarbital for 24 hrs; (C) same as (A) except 1 mM methyropone was present; and (D) same as (A) except mM SKF-525A was present. For these inhibitors, cells were preincubated on ice for 30 min before adding the drug and the spin trap. The ESR settings were similar to those described in Figure 1.

derivatives and that phenobarbital inducible form of cytochrome P-450 is responsible for this free radical-dependent activation. Our study also indicated that human tumor cells as well as hepatic cells are capable of metabolizing these hydrazine derivatives to free radical species. However, it is quite interesting that procarbazine is metabolized in HL60 tumor cells by peroxidases and appears to be different, at least in this cell line, than iproniazid which is mostly metabolized by cytochrome P450. In hepatic cells, however, both of these drugs were metabolized by cytochrome II-450. It is also interesting to note that we were unable to detect benzyl radical from procabazine as we previously detected using horseradish peroxidase.

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