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METABOLIC ACTIVATION OF HYDRALAZINE BY RAT LIVER MICROSOMES*

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Abstract—There is evidence to suggest that the oxidative metabolism of hydralazine (HP), an antihypertensive drug, may represent a toxic pathway which could account for some of the adverse effects of the drug. Experiments were done to determine whether the hepatic oxidative metabolism of HP is associated with the formation of reactive metabolites. In the presence of NADPH, HP was metabolized by rat liver microsomes to three major oxidation products, phthalazine, phthalazinone (PZ), and a dimer compound. Under similar incubation conditions, radioactivity derived from [^{14}C]HP was covalently bound to microsomal protein. Metabolite formation and covalent binding increased following pretreatment of rats with phenobarbital. In contrast, pretreatment with 3-methylcholanthrene or with the monooxygenase inhibitor, piperonyl butoxide, slightly decreased both metabolite formation and covalent binding. Electron spin resonance (ESR) analyses indicated that nitrogen-centered radicals were formed when rat liver microsomes were incubated with HP under conditions similar to those required for covalent binding and for the production of the oxidative metabolites. In addition, reduced glutathione (GSH) caused concentration-dependent decreases in the production of phthalazine, PZ, and the dimer, in the covalent binding of HP to microsomal protein, and in the formation of nitrogen-centered radicals. The results of these investigations indicate that the oxidative metabolism of HP by rat liver microsomes is highly correlated with the formation of nitrogen-centered radicals and the production of metabolites that become covalently bound to microsomal protein. These observations support the hypothesis that the oxidation of HP generates reactive metabolites which may contribute to the toxicity of the drug.

The major side effect associated with the use of hydralazine (HP), an antihypertensive agent, is a syndrome closely resembling the autoimmune disease, systemic lupus erythematosus. The pathogenesis of HP-induced lupus is not understood completely. An important predisposing factor is the slow acetylator phenotype [1]. HP-induced lupus occurs predominantly in individuals with a low capacity for N-acetylation, an important metabolic step in the elimination of HP from the body. Another major pathway for the metabolism of hydrazine derivatives is N-oxidation, and there is some evidence to suggest that the oxidative metabolism of HP may be involved in the adverse effects of the drug. For example, it has been demonstrated that phthalazinone (PZ), an oxidation product of HP, is excreted in greater amounts by slow acetylators than by fast acetylators [2–4]. Thus, increased PZ formation, like slow acetylator status, is correlated with development of the disease.

Streeter and Timbrell [5, 6] have reported recently that the metabolism of HP by rat liver microsomes leads to the formation of PZ and phthalazine and of reactive metabolites which covalently bind to microsomal protein. We confirmed the findings of Streeter and Timbrell and, in addition, found several other metabolites including a major oxidative product that was identified as a dimerization product of phthalazine and 1-aminophthalazine (Fig. 1) [7]. Formation of the dimer provides indirect evidence that free radical intermediates are produced during the microsomal metabolism of HP. However, very little is known about the nature of the reactive metabolites produced or the specific pathway(s) involved in the activation of HP. The objective of the investigations

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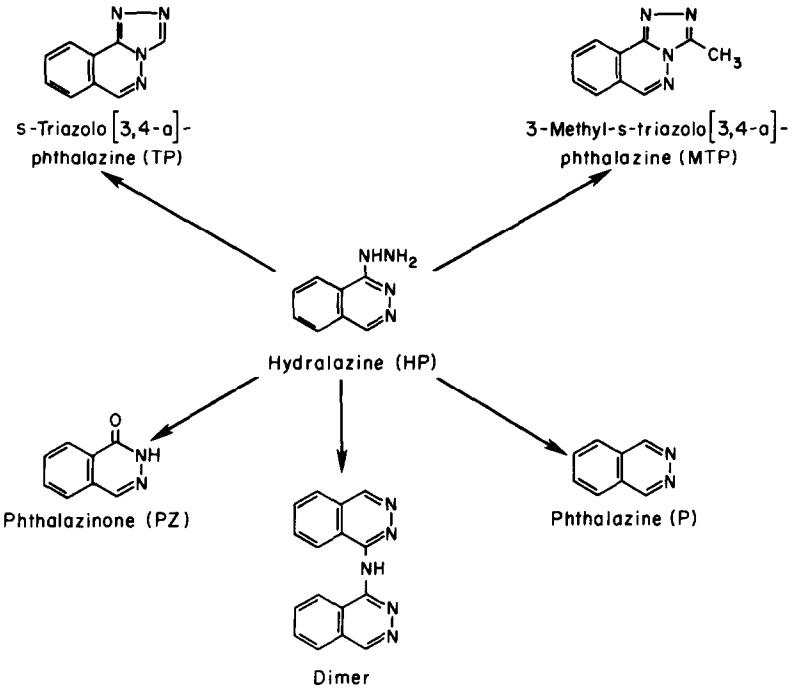

Fig. 1. Metabolites produced by incubation of rat liver microsomes with hydralazine in the presence of NADPH as described in Materials and Methods.

described in this communication was to pursue further the mechanisms involved in the activation of HP by rat liver microsomes. Activation of HP was monitored by covalent binding to microsomal protein and by the generation of free radical species as detected by electron spin resonance (ESR).

MATERIALS AND METHODS

Chemicals. Hydralazine hydrochloride was supplied by the Ciba-Geigy Co. (Summit, NJ). [^{14}C]Hydralazine hydrochloride (6.77 mCi/mmol; radiochemical purity >99%) was obtained from the California Bionuclear Corp. (Sun Valley, CA). Phthalazine, phenacetin, and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). DMPO was purified prior to use with neutral decolorizing charcoal by the method of Beuttner and Oberly [8]. Phthalazinone (PZ) was purchased from Pfaltz & Bauer Research Chemicals (Stamford, CT). *s*-Triazolo[3,4-*a*]phthalazine (TP) and 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP) were prepared by previously published procedures [9, 10]. The purity of the compounds was confirmed by HPLC. NADPH, reduced glutathione (GSH), 3-methylcholanthrene, benzo[*a*]pyrene, and horseradish peroxidase were obtained from the Sigma Chemical Co. (St. Louis, MO). Ethylmorphine was obtained from Merck & Co., Inc. (Rahway, NH). Phenobarbital was purchased from the Ruger Chemical Co. (Irvington-on-Hudson, NY) and piperonyl butoxide was obtained from ICN Pharmaceuticals, Inc. (Plainview, NY). Reagent grade inorganic materials and HPLC grade organic solvents were obtained from the Fisher Scientific Co. (Pittsburgh, PA).

Animals. Adult, male Sprague–Dawley rats (200–224 g), obtained from Hilltop Laboratory Animals (Scottdale, PA), were used in all experiments. Animals were maintained under standardized conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°). Food (Wayne Laboratory Chow) and water were provided *ad lib*. In some experiments, animals were injected intraperitoneally with sodium phenobarbital in saline (80 mg/kg) daily for 3 days, 3-methylcholanthrene in cottonseed oil (40 mg/kg) daily for 2 days, or piperonyl butoxide (800 mg/kg) 1.5 hr prior to sacrifice. The control rats were treated with the appropriate vehicle for the same periods of time.

Incubation conditions. Rat liver microsomes were prepared as described in a previous report [11]. Hepatic microsomal suspensions were incubated with 0.05 M Tris–HCl (pH 7.4), 5.0 mM $MgCl_2$, NADPH (0.5 mM), and microsomal protein in a total volume of 2.0 ml. Incubations were done in 25-ml Erlenmeyer flasks at 37° under air in a Dubnoff metabolic incubator. The reaction was initiated by the addition of HP (4 mM) or [^{14}C]HP (40 μM; 6.77 mCi/mmol) dissolved in $MgCl_2$–Tris buffer. Tissue concentrations and incubation times were chosen to assure linearity of product formation with time. In some experiments, incubations were carried out in the absence of NADPH or tissue, or in the presence of GSH (0.2 to 10.0 mM). For the ESR studies, DMPO (100 mM) was added to the medium prior to incubation. Where indicated, microsomes obtained from rats pretreated with monooxygenase inducing agents or inhibitor were employed. The

extent of induction or inhibition was monitored by measuring the activities of ethylmorphine demethylase and benzo[*a*]pyrene hydroxylase and by determining the cytochrome P-450 content of the microsomal preparations [12–14]. Heat-treated microsomes were prepared by heating microsomal suspensions at 70° for 3 min. After heating, there was no detectable NADPH–cytochrome *c* reductase activity [15].

In some studies, horseradish peroxidase was dissolved in 0.1 M phosphate buffer (pH 7.4) to give a final concentration of 0.5 mg/ml. Samples of 2 ml were added to 25-ml Erlenmeyer flasks and placed on ice. Where indicated, DMPO (100 mM) and hydrogen peroxide (400 μM) were added, the reaction was initiated by the addition of HP (4 ml), and incubations were carried out as described above. In all experiments, HP metabolites were isolated and quantified by the HPLC method previously described [16].

Covalent binding to microsomal protein. After incubation with [^{14}C]HP, the contents of the incubation flasks were added to 1 ml of 15% trichloroacetic acid. The protein was precipitated by centrifugation and the pellet was washed with 5 ml each of 80% methanol once, ethanol–ether (3:1) three times, and with 80% methanol once more. After the final wash, radioactivity was not detectable in the supernatant fraction. The final pellet was redissolved in 1 ml of 1.0 N sodium hydroxide at 70° for 5 min and added to 10 ml of ScintiVerse II (Fisher Scientific Co.) scintillation fluid. Radioactivity was measured with a Beckman LS 9000 scintillation spectrometer (Beckman Instruments, Inc., Fullerton, CA). Protein concentration was determined by the method of Lowry *et al.* [17].

Electron spin resonance. A spin trapping technique was used to facilitate the measurement of free radical species derived from HP. DMPO was the spin trapping agent used, and analyses were done in an ESR quartz flat cell (Wilmad Glass Co., Buena, NJ). Approximately 200 μl of the incubation mixture was used for analysis, and the ESR spectra were recorded on a Bruker ER 200-D spectrometer equipped with a Bruker Aspect 2000 computer (Bruker-Physik AG, Karlsruhe, West Germany). The instrument settings are described in Results for each spectrum illustrated.

RESULTS

When [^{14}C]HP was incubated with rat liver microsomes in the presence of NADPH, radioactivity became bound to microsomal protein and could not be removed by repeated washings with organic solvents. In the absence of NADPH, the amount of binding decreased by approximately 80% (Fig. 2). The NADPH-dependent binding was linear with incubation time for approximately 15 min; consequently, an incubation time of 10 min was chosen for all subsequent experiments. The NADPH-dependent binding increased by approximately 30% following pretreatment of rats with phenobarbital. In contrast, pretreatment with 3-methylcholanthrene or with the monooxygenase inhibitor, piperonyl butox-

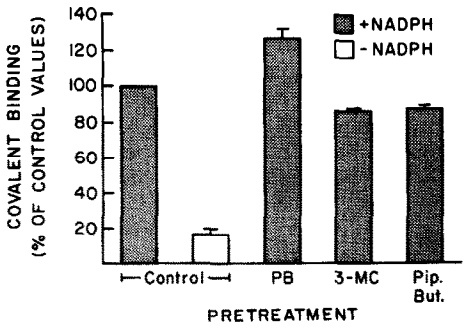

Fig. 2. Enzyme-mediated covalent binding by hydralazine to rat liver microsomal protein. [^{14}C]Hydralazine (40 μM) was incubated for 10 min with rat liver microsomes (1 mg protein/ml) obtained from control animals or from animals pretreated with phenobarbital (PB), 3-methylcholanthrene (3-MC), or piperonyl butoxide (Pip. But.), and covalent binding was determined as described in Materials and Methods. Each value is expressed as the mean percent of control ± SE of three experiments. The control value in the presence of NADPH was 0.24 ± 0.005 nmol · mg protein^{-1} · min^{-1}.

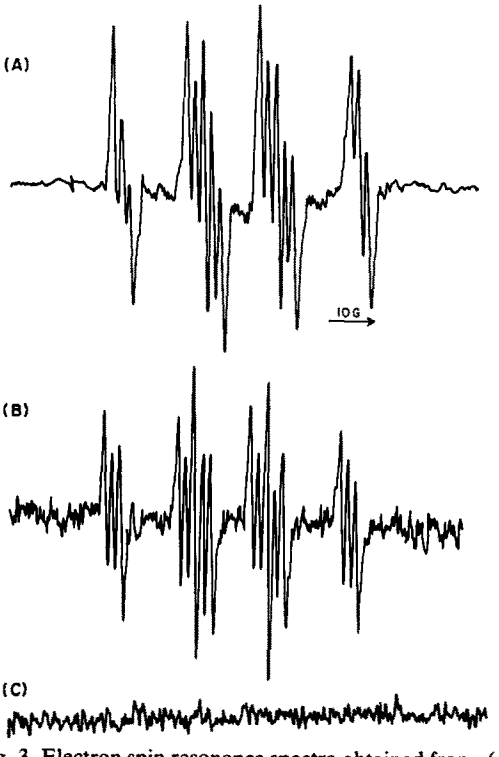

Fig. 3. Electron spin resonance spectra obtained from: (A) incubation of hydralazine (4 mM) with rat liver microsomes (4 mg protein/ml), NADPH (0.5 mM), and DMPO (100 mM), with $a^N = 15.94$ G, $a^H_\beta = 19.73$ G, $a^N_\beta = 1.89$ G. The instruments settings were: microwave power, 63 mW; modulation amplitude, 1.25; gain, 8×10^4; scan range, 100 G; time constant, 5000 msec; scan time, 1000 sec. (B) Incubation of hydralazine (4 mM) with horseradish peroxidase (0.5 mg/ml), H_2O_2 (400 μM), and DMPO (100 mM), with $a^N = 15.94$ G, $a^H_\beta = 19.46$ G, $a^N_\beta = 1.62$ G. The instrument settings were: microwave power, 63 mW; modulation amplitude, 0.4; gain, 5×10^5; scan range, 100 G; time constant, 200 msec; scan time, 200 sec. (C) Same incubation conditions as (A) or (B) except that hydralazine was omitted or GSH (10.0 mM) was added. The instrument settings were identical to (B).

ide, caused a slight decrease in the binding of radioactivity to microsomal protein (Fig. 2).

Electron spin resonance studies indicated that when rat liver microsomes were incubated with HP and the spin trapping agent, DMPO, in the presence or absence of NADPH, a 16-line spectrum was produced with hyperfine splitting constants, $a^N = 15.94$ G, $a_\beta^H = 19.73$ G, $a_\beta^N = 1.89$ G (Fig. 3). This spectrum is indicative of a nitrogen-centered DMPO adduct. When heat-treated microsomes were used, a weak 16-line spectrum was barely detectable. When horseradish peroxidase was incubated with HP and H_2O_2 in the presence of DMPO, a 16-line spectrum similar to that produced by rat liver microsomes was observed. In the absence of H_2O_2, a weak, poorly resolved 16-line spectrum was detected. In the absence of tissue or of HP, no ESR spectrum was observed.

Addition of GSH (1.0 to 10.0 mM) to incubation flasks containing rat liver microsomes, HP, and NADPH caused a concentration-dependent decrease in formation of the three oxidative metabolites, phthalazine, PZ, and the dimer (phthalazine and 1-aminophthalazine) (Fig. 4). In contrast, GSH had no effect on the production of TP, a product of non-oxidative pathways (Fig. 4). Similarly, when rat liver microsomes were incubated with [^{14}C]HP in the presence of NADPH, concentrations of GSH ranging from 25 to 500 μM caused a concentration-dependent decrease (30–80%) in the amount of radioactivity which became covalently bound to microsomal protein. In addition, GSH (10.0 mM) blocked the formation of the ESR spectrum resulting from incubation of rat liver microsomes with HP, NADPH, and DMPO as well as that produced by incubation of horseradish peroxidase with HP, H_2O_2, and DMPO (Fig. 3).

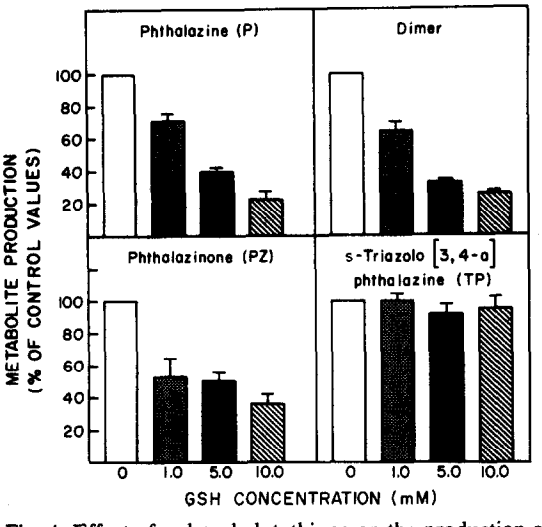

Fig. 4. Effect of reduced glutathione on the production of the major oxidative metabolites of hydralazine. Hydralazine (4 mM) was incubated for 20 min with rat liver microsomes (4 mg protein/ml) in the presence of NADPH (0.5 mM). Metabolites were separated and quantified by HPLC as described in Materials and Methods. Each value is expressed as the mean percent of control ± SE of four experiments. The control values for phthalazine, phthalazinone and TP were: 0.40 ± 0.026, 0.06 ± 0.004, and 0.11 ± 0.008 nmol · mg protein^{-1} · min^{-1} respectively. For the dimer, a chemical standard was not available; therefore we used the HPLC peak area ratio of the u.v. absorbance of the dimer and internal standard, as previously described [16].

DISCUSSION

Previous studies suggested that metabolic activation of HP via oxidative pathways might contribute to the adverse effects of the drug. The results presented in this report and those of Streeter and Timbrell [6] indicate that rat liver microsomes catalyze the NADPH-dependent conversion of HP to a reactive metabolite(s) capable of covalent bond formation to microsomal protein. We found that phenobarbital pretreatment significantly increased the NADPH-dependent binding. In contrast, pretreatment with the inducing agent, 3-methylcholanthrene, or with the monooxygenase inhibitor, piperonyl butoxide, decreased the binding. The effects of the inducing agents and the inhibitor on the covalent binding of HP paralleled their effects on the formation of the three major oxidation products, phthalazine, PZ and the dimer [7]. These results suggest that phenobarbital-inducible forms of cytochrome P-450 may be involved in the microsomal metabolism and activation of HP. In contrast to these observations, Streeter and Timbrell previously reported that phenobarbital pretreatment has no effect on the NADPH-dependent covalent binding of HP, whereas, 3-methylcholanthrene pretreatment slightly increases binding [6]. The reason for the difference between their findings and ours is not known. Like Streeter and Timbrell, we found that GSH caused concentration-dependent decreases in both the formation of the oxidation products of HP and in the covalent binding. These observations are consistent with the hypothesis that a reactive intermediate(s) is formed during the oxidative metabolism of HP.

The results of the ESR experiments indicate that the reactive microsomal metabolite of HP involved in the covalent binding may be a nitrogen-centered radical. The DMPO adduct was produced under incubation conditions similar to those required for oxidative metabolite formation and covalent binding. The formation of a nitrogen-centered radical in the absence of NADPH was not totally unexpected, since there was also some measurable covalent binding as well as metabolite formation in the absence of NADPH. These results are probably attributable to the chemical degradation of HP known to occur in aqueous solutions of neutral to alkaline pH [18]. A nitrogen-centered DMPO adduct, similar to that produced by hepatic microsomes, was detected during HP incubation with horseradish peroxidase, confirming the findings of Sinha [19]. The three oxidation products produced by rat liver microsomes are also formed during the incubation of horseradish peroxidase with HP [7], suggesting that a similar free radical oxidation pathway is involved in both systems.

The identity of the nitrogen-centered DMPO adduct is not known. A mechanism for the formation

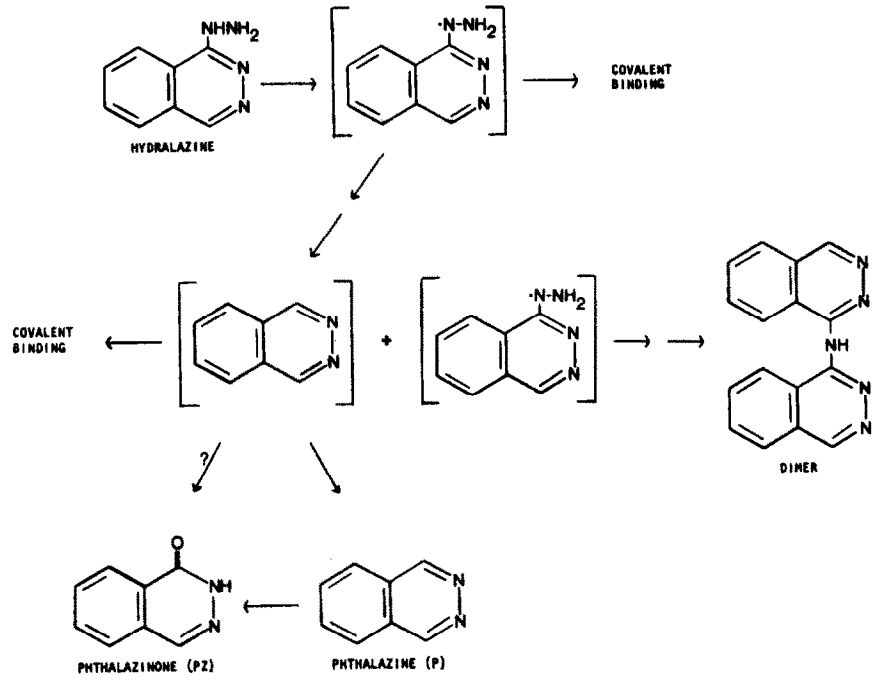

Fig. 5. Proposed pathways for the metabolic activation of hydralazine.

of a nitrogen-centered radical derived from HP may be analogous to that proposed by Misra and Fridovich [20] for the free radical oxidation of phenylhydrazine. This mechanism involves the two electron oxidation of HP and proceeds through several intermediates, including the nitrogen-centered phthalazinylhydrazyl (RNNH) and diazene (RN=NH) radicals. The diazene radical decomposes to molecular nitrogen (N_2) and a carbon-centered phthalazinyl (R·) radical. This pathway includes two different nitrogen-centered radicals and provides a mechanism for the formation of phthalazine, PZ, and the dimer via free radical intermediates. Proposed pathways for the activation and subsequent metabolism of HP are summarized in Fig. 5. Although a carbon-centered radical was not trapped by DMPO in this study, its formation cannot be excluded. The success of spin trapping experiments depends on the kinetic conditions that exist in the system. For a favorable rate of spin adduct formation, the rate of the spin trapping reaction must be greater than the rates of other reactions for the radical. In this case, the carbon-centered radical may react too slowly with DMPO for detection by ESR. In addition, the phthalazinyl radical may be rapidly consumed in the formation of phthalazine and the dimer. The decrease in metabolite formation in the presence of GSH provides indirect evidence for the existence of this radical intermediate.

The studies presented in this report demonstrate that there is a correlation among the microsomal production of HP oxidation products, the generation of nitrogen-centered radicals, and the covalent binding of HP metabolites to microsomal protein. Therefore, the oxidative metabolism of HP may be an activation pathway for the drug. Since drug-induced lupus is a systemic disease, the metabolic activation of HP may also be catalyzed by enzymes similar to horseradish peroxidase, such as prostaglandin synthetase, which is found in almost all mammalian cell types [21]. It has been demonstrated that HP can be metabolized to nitrogen-centered radicals by prostaglandin synthetase [19]. In addition, red blood cells convert HP to nitrogen-centered radicals [22], and we have found that HP is metabolized by red blood cells to phthalazine, PZ, and the dimer (data not shown), suggesting that metabolism similar to that catalyzed by hepatic microsomal enzymes may occur in red cells. The oxidative pathway may be enhanced in phenotypically slow acetylators of HP and, therefore, could be involved in the pathogenesis of HP-induced lupus.

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