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INHIBITION OF NADPH-CYTOCHROME P450 REDUCTASE BY CYCLOPHOSPHAMIDE AND ITS METABOLITES[1]

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SUMMARY:

Cyclophosphamide (CP) administration to rats produced a dose-dependent loss of hepatic NADPH-cytochrome-P450 reductase and microsomal mixed function oxidase (MFO) activities. In vitro CP, its metabolites (acrolein, phosphoramide mustard, 4-keto CP and nor-nitrogen mustard) and Ifosfamide, which is an analog of CP, were tested for their effects on the reductase activity. Only acrolein produced a significant loss of the reductase (66%). This loss of activity could be prevented by the presence of cysteine in the incubation mixture. Acrolein also produced a dose dependent loss of the activity when incubated with the purified reductase. These data suggest that CP-induced loss of the reductase results from interaction between CP metabolite acrolein and critical sulfhydryl groups in the reductase.

INTRODUCTION:

Since its introduction, cyclophosphamide (CP) has proved to be an effective chemotherapeutic agent against a variety of animal and human tumors (1,2). In addition, it has been widely used as an immunosuppressive agent in the treatment of various non-malignant diseases (3,4). CP is a prodrug which requires metabolic activation by the hepatic microsomal mixed function oxidase

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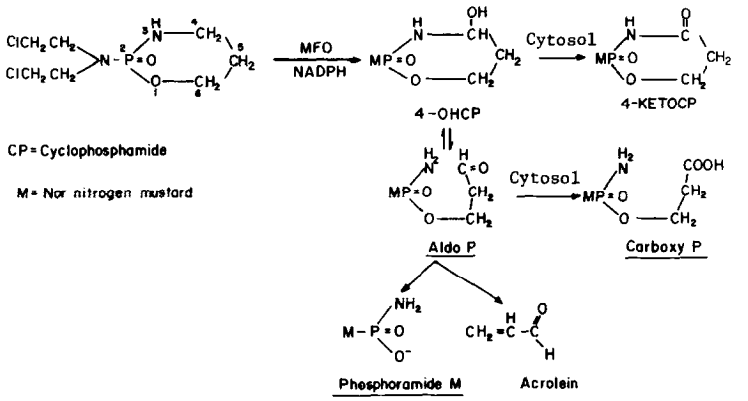

Figure 1. Metabolism of Cyclophosphamide by Hepatic Microsomal and Cytosolic Enzymes.

MFO, mixed function oxygenase.

(MFO) complex in order to exert its effects (Figure 1). In the rat, high doses of CP inhibit a number of MFO-associated enzyme activities including arylhydrocarbon hydroxylase (AHH) and aminopyrine demethylase (5,6). We have recently reported that CP produces a significant loss of microsomal cytochrome P450 content and that this is due to the interaction of the CP metabolite acrolein with cytochrome P450 (7).

Cytochrome P450 is the terminal oxidase of the electron transport chain of the MFO in liver and other organs. An integral component of this enzyme complex is the enzyme NADPH-cytochrome P450 reductase (EC 1.6.2.4) which catalyzes the transfer of electrons from NADPH to cytochrome P450 and to other electron acceptors such as cytochrome c (8,9). Considering the intimate association between cytochrome P450 and the reductase, it is possible that any perturbation of this relationship may alter the efficiency of drug metabolism. Since CP produces a significant loss of cytochrome P450 (7), it was of interest to investigate the effect of CP on the reductase. In this report we present evidence which shows that CP causes a significant loss of

the reductase activity and the CP metabolite responsible for this loss has been tentatively identified.

MATERIALS AND METHODS

Chemicals:

CP, ifosphamide and phosphoramide mustard were obtained from the Drug Development Branch of NCI Bethesda, MD. Acrolein was obtained from Eastman Kodak, Rochester, NY. 2'-AMP and agarose-hexane-adenosine 2',5'-diphosphate were obtained from Sigma Chemical Company, St. Louis, Missouri and P.L. Biochemical, Milwaukee, Wis., respectively. The sources of the materials used during the enzyme purification have been previously reported (10).

Animal Treatment:

Male Sprague-Dawley rats (200-250 g), obtained from Harlan Industries, Indianapolis, Indiana, were used in all studies. All animals were maintained under identical conditions and received food and water ad libitum. For in vivo studies, groups of rats (4 rats/group) received different doses of CP, given i.p. in saline, and the animals were sacrificed on day 4 for the isolation of hepatic microsomes. For in vitro studies, hepatic microsomes were isolated from rats that had received phenobarbital (0.1%) in drinking water for 6 days prior to sacrifice. Hepatic microsomes were isolated by previously reported methods (6,7) and protein was determined by the method of Lowry et al. (11), using bovine serum albumin as the standard.

Preparation of Purified NADPH-Cytochrome P450 Reductase:

The reductase was purified according to the procedure of Yasukochi and Masters (12) as subsequently modified by Guengerich (10, 13). The purified protein migrated as a single band in SDS-polyacrylamide gel electrophoresis system and had an apparent M_R of 72,000. The specific activity of the enzyme was 25000 nmole cytochrome c reduced $min.^{-1}mg^{-1}$.

Measurement of NADPH-Cytochrome P450 Reductase Activity:

The reductase was assayed at 22° in 0.3 M KPO_4 buffer (pH 7.7) by the previously reported methods, using Cytochrome C as the electron acceptor (9,12).

Interaction of CP Metabolites with NADPH-Cytochrome P450 Reductase:

A typical incubation in a total volume of 1.0 ml contained: 100 µmoles KPO_4 pH 7.4, 5.4 mg microsomal protein and 1.0 µmole CP or its metabolite or the analog. The mixture was incubated for 30 min at 37°C. An aliquot of this mixture was used to measure the reductase activity. When purified reductase was substituted for microsomes, the volume of the incubation was reduced to 0.5 ml and contained 0.026 mg purified reductase protein. In some cases, various concentrations of cysteine were included in the incubation mixture. Control incubations contained all components except CP (its metabolites or the analog).

Cytochrome P450 Spectrum:

Cytochrome P450 was determined by difference spectroscopy. Briefly, 3 ml of the treated microsomal suspension was transferred to both the reference and sample cuvettes and a base line of equal light absorbance was recorded. A few

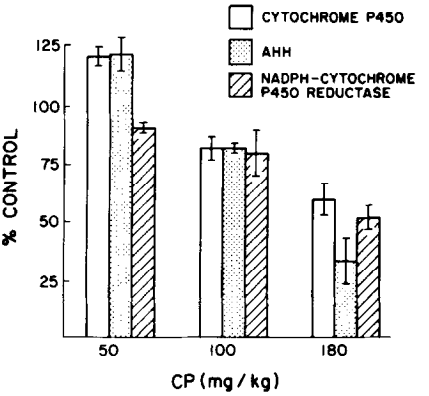

Figure 2. Dose Dependent In Vivo Inhibition of the Hepatic NADPH-Cytochrome P450 Reductase and the Microsomal Mixed Function Oxidase Enzymes by Cyclophosphamide.

Animals received various doses of CP or vehicle (saline) on Day 0. On day 4, animals were sacrificed and microsomes isolated. Assays were performed as described in the Materials and Methods.

mg of dithionite were added to both cuvettes. Subsequently, carbon monoxide was bubbled into the sample cuvette for 30 seconds. The difference spectrum was recorded using an Aminco DW-2 recording spectrophotometer and cytochrome P450 content was calculated using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ (14).

Measurement of AHH Activity:

Aryl hydrocarbon hydroxylase (AHH) activity was measured as previously reported (15). Benzo(a)pyrene was used as the substrate and the formation of phenolic metabolites was quantitated by measurement of their fluorescence using 396 nm wavelength for excitation and 522 nm wavelength for emission. Fluorescence was compared with a standard curve obtained with 3-hydroxybenzo (a)pyrene. AHH activity is expressed as pmole equivalent of 3-hydroxybenzo(a)pyrene produced per mg protein during a 10-minute incubation at 37°. An Aminco-Bowman spectrophotofluorometer employed in these studies was calibrated with a quinine sulfate standard before and during each determination.

RESULTS AND DISCUSSION

As shown in Figure 2, CP administration to rats produced a dose dependent loss of the reductase. At a low dose of CP (50 mg kg^{-1}), the loss of the reductase was about 8% but the levels of cytochrome P450 and AHH were somewhat elevated (20%) above the control values. An intermediate dose of CP (100 mg kg^{-1}) produced a 25% inhibition of all the activities tested. However, a

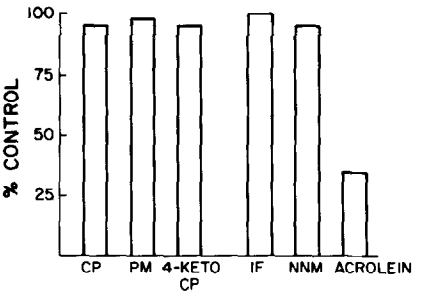

Figure 3. Inhibition of the Microsomal NADPH-Cytochrome P450 Reductase by CP and its Metabolites.

Microsomes were incubated with 1.0 mM of each chemical for 30 minutes at 37°C. Assays for reductase activity were performed as per Materials and Methods. Abbreviations are as follows: CP, cyclophosphamide; PM, phosphoramide mustard; 4-keto CP, 4-keto cyclophosphamide; IF ifosfamide; NNM, nor-nitrogen mustard.

higher dose of CP (180 mg kg^{-1}) produced a greater inhibition of the enzymes: the reductase activity was reduced by 48%, while the levels of cytochrome P450 and AHH were decreased by 40% and 67%, respectively.

In order to determine whether the inhibition was dependent on the metabolism of CP and if so which metabolite(s) of CP was responsible for this inhibition, we tested the effects of CP, its metabolites and an analog on the reductase in an *in vitro* incubation (in absence of NADPH) containing microsomes and the test chemical. Of all the compounds studied (CP, acrolein, 4-keto CP, phosphoramide mustard, nor-nitrogen mustard and ifosfamide), only acrolein caused a significant loss of the reductase (Figure 3). In different experiments, acrolein (1mM) produced a 50-66% loss of the reductase activity, while equimolar concentrations of the other compounds caused $\leq$ 5% loss.

The data presented in Table 1 show that acrolein also caused a dose-dependent loss in the activity of the purified reductase. The inhibition ranged from 43% at 0.6 mM acrolein to 86% at 1.5 mM. At equivalent concentrations of acrolein, the purified reductase was 40% more sensitive than the crude microsomal reductase (data not shown).

TABLE I

Inhibition of purified NADPH cytochrome P450 reductase by CP metabolite, acrolein. Incubations and assays were performed as described in Materials and Methods.

mM Acrolein	% Activity as
0	100
0.45	84
0.6	57
0.9	43
1.5	13

The *in vitro* inhibition of the reductase by acrolein was effectively blocked by the addition of cysteine to the incubation. As shown in Figure 4, acrolein (1mM) produced a 50% loss of the reductase which was counteracted by increasing the concentration of cysteine such that an equimolar concentration of cystein (1mM) completely blocked the inhibition by acrolein.

The data presented suggest that the inhibition of NADPH-cytochrome P450 reductase by CP is a result of the interaction of the CP metabolite acrolein with critical sulfhydryl groups in the flavoprotein. We have proposed a simi-

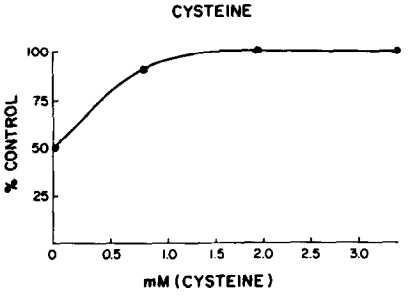

Figure 4. Protection by Cysteine of the *In Vitro* Inhibition of the NADPH-Cytochrome P450 Reductase by CP Metabolite, Acrolein.

Microsomes were incubated with acrolein (1 mM) alone or with acrolein plus varying concentrations of cysteine. Incubations were done at 37°C for 30 min. An aliquot of the incubation mix was assayed for the reductase activity.

lar mechanism to explain the significant denaturation of cytochrome P450 associated with CP metabolism (7,16). In both cases, cysteine affords complete protection against acrolein-mediated inhibition. It has been reported that the enzymatic integrity of the reductase requires the presence of functional cysteinyl residues in the protein (17). Covalent modification of cysteine residues by sulfhydryl reagent p-chloromercuribenzoate results in significant inhibition of the reductase (18,19). Although it has been reported that protease solubilized purified reductase (isolated from pig liver) contains approximately 6 mol thiol groups/mol protein (19), the exact location and function of these groups is not clear. Ebel reported that covalent modification of arginine residues by 2,3-butanedione also results in a significant loss of the reductase (20), so it is likely that residues other than cysteine may be involved in the destruction of the reductase. However, our present data, as well as those on cytochrome P450 (7,16), suggest that acrolein causes loss in the activity of the reductase by altering the integrity of critical sulfhydryl groups in the flavoprotein.

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