Quantitative Studies on the In Vitro Metabolic Activation of Dimethylnitrosamine by Rat Liver Postmitochondrial Supernatant

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The metabolic activation of dimethylnitrosamine (DMN) to mutagenic and/or cytotoxic intermediates in vitro has been characterized and the relationship between DMN demethylase and ethoxyresorufin-O-deethylase (EROD) or ethylmorphine-N-demethylase (EMND) has been evaluated. A mammalian assay system which uses the postmitochondrial supernatant (S-15 fraction) prepared from a rat liver homogenate as an enzyme source and V79 Chinese hamster cells as targets for chemically induced damage was used. The enzyme pattern of the S-15 fraction was altered by pretreatment of experimental animals in vivo and/or by the use of enzyme inhibitors in vitro.

The results of these studies indicate that the concentration of S-15 fraction in the reaction mixture can markedly influence the degree of DMN-induced cytotoxicity when it is metabolized in vitro and that the degree of DMN-induced cytotoxicity and mutagenicity are linearly related. The degree of cytotoxicity and mutagenicity induced in V79 cells by DMN does not correlate with EROD activity (a measure of 3-methylcholanthrene-inducible mixed-function oxidases) nor with EMND activity (a measure of phenobarbital-inducible mixed function oxidases) in the S-15 fraction.

Introduction

The metabolic activation system incorporated into short-term mutagenicity assays usually consists of a hepatic postmitochondrial supernatant (PMS) along with selected cofactors. The mixed-function oxidase system in a hepatic PMS is known to consists of multiple cytochrome P-450 isozymes with different, but overlapping substrate specification. The ability of a given PMS enzymatically to deethylate ethoxyresorufin or to demethylate ethylmorphine is often used to characterize the profile of cytochrome P-450 present.

Although each of these reactions is likely to be catalyzed by more than one isozyme of cytochrome P-450, the rate of each reaction is clearly a marker for a different pattern of mixed function oxidase activity. Ethoxyresorufin-O-deethylase (EROD) activity is considered to be a measure of 3-methylcholanthrene(MC)-inducible mixed-function oxidase activity, while ethylmorphine-N-demethylase (EMND) activity is considered to be a measure of phenobarbital-inducible mixed-function oxidase activity. The relationship between these widely used mixed-function oxidase “marker activities” and the ability to metabolically activate carcinogens and mutagens is poorly understood.

Dimethylnitrosamine (DMN) is a hepatocarcinogenic nitrosamine (1,2) that requires metabolic activation in order to function as a mutagen (3-6), although at high concentrations DMN is cytotoxic in the absence of metabolic activation due to its protein denaturing ability, which may result in cell membrane destruction (7).

The metabolic activation of DMN is catalyzed by (an) enzyme(s) termed DMN demethylase (8). The demethylase activity in an in vitro metabolic activation system correlates, in some cases, with DMN-induced mutagenicity (9-11), whereas in other instances DMN demethylase activity does not correlate with DMN-induced mutagenesis (12). Isolated microsomes from mouse livers exhibit only about half the DMN demethylase activity present in the crude homogenate (13-15). The enhancing effect of the soluble fraction may be due to the presence of additional cofactors for the microsomal enzymes because the hepatic soluble fraction is unable to metabolize DMN in the absence of microsomes (14-16). Recent evidence suggests that DMN demethylase may be composed of several mixed-function oxidases (17-19). The objective of the experiments reported here was to determine whether the cytochrome P-450 isozymes which comprise DMN demethylase activity correlate with EMND activity or with EROD activity.
Materials and Methods

Materials

Calf serum, antibiotics and culture medium were purchased from Gibco (Grand Island, NY). Ouabain, NADPH, NADP, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-methylcholanthrene, trypsin, dimethyltryanosine, metyrapone, α-naphthoflavone and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). β-Naphthoflavone was from Aldrich Chemical Co. (Milwaukee, WI) phenobarbital from Mallinckrodt (St. Louis, MO), and Giemsa stain (improved R66 solution) from Biomedical Specialties (Santa Monica, CA). Aroclor 1254 brand of polychlorinated biphenyls (PCB) (Monsanto Chemical Co., St. Louis, MO) was a gift from J. B. Hook, Michigan State University.

Cell Line and Culture Conditions

The cells used in these experiments are V79 Chinese hamster cells that grow in monolayer (obtained as a gift from J. E. Trosko and C. C. Chang, Michigan State University). This cell line was derived from the lung of a normal, male Chinese hamster (20). The cells are routinely cultured at 37°C in humid air containing 5% CO2, in modified Eagle's minimal essential medium (Gibco formula 78-5470) supplemented with 20% (v/v) calf serum, penicillin (100 units/mL), streptomycin (100 µg/mL), fungizone (0.25 µg/mL), sodium pyruvate (110 µg/mL), glucose (1mg/mL), sodium chloride (0.83 mg/mL) and sodium bicarbonate (1 mg/mL).

Preparation of Hepatic Postmitochondrial Supernatant (S-15 fraction)

Male Sprague-Dawley rats (Spartan Research Farms, Haslett, MI) weighing 180 to 240 g were maintained on a 12 hr light cycle (7 p.m.-7 a.m.), allowed free access to food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and water, and were acclimated to our animal quarters for at least 3 days prior to use.

Groups of animals were pretreated with IP injections of MC (80 mg/kg body weight in peanut oil, 24 hr prior to sacrifice), BNF (80 mg/kg body weight in peanut oil, 24 hr prior to sacrifice), Aroclor 1254 brand of PCB (500 mg/kg body weight in peanut oil, 96 hr prior to sacrifice) or phenobarbital (80 mg/kg body weight in isotonic NaCl solution, 96, 72, 48 and 24 hr prior to sacrifice). Controls received vehicle only.

The hepatic postmitochondrial supernatant is prepared (5) as aseptically as possible, at 0 to 4°C, just prior to use. Animals are killed by a sharp blow to the back of the head followed by cervical dislocation. The liver is removed, washed with 0.9% saline, and homogenized with a Potter-Elvehjem type homogenizer in 3 volumes of sucrose-HEPES buffer (0.25 M sucrose containing 2mM MgCl2 and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4). The homogenate then undergoes two successive centrifugations (Sorvall RC 2-B refrigerated centrifuge); 9000 g for 10 min. followed by 15,000 g for 20 min. The postmitochondrial supernatant obtained is referred to as the S-15 fraction.

Cytotoxicity and Mutagenicity Assays

The assays were performed in the following manner. Stock cultures of V79 cells in the midlogarithmic phase of growth were trypsinized (0.01% crystalline trypsin dissolved in phosphate-buffered saline, pH 7.8, 37°C; 10 min) and the cell number determined with a hemocytometer. The cells are diluted to the desired concentration and seeded into 10 mL of warm (25°C) growth medium in 100-mm tissue culture dishes. The number of cells seeded per dish is 200 to 2000 for cytotoxicity assessment and 1 × 105 to 5 × 105 for the determination of mutation frequency. The actual number is dependent upon the anticipated toxicity of a particular treatment. The same stock population of V79 cells is used for parallel determinations of cytotoxicity and mutation frequency.

The cells are allowed 6 hr to attach to the culture dishes. The growth medium is then removed and the treatment medium is added. This consists of the following components, added in order: (1) 5.9 mL phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.15 g Na2HPO4, 0.2 g KH2PO4/L glass-distilled water, pH 7.3); (2) 2.0 mL of the cofactor mixture, composed of the following (final concentration in the reaction mixture) dissolved in 66 mM Tris-HCl, pH 7.5: NADPH, 0.1 mM; NADP, 0.3 mM; NADH, 0.4 mM; MgCl2, 3.0 mM; glucose-6-phosphate, 4.2 mM; and glucose-6-phosphate dehydrogenase, 1 unit/mL; (3) 2.0 mL of the S-15 fraction, diluted with sucrose-HEPES buffer to the desired final protein concentration in the treatment medium; (4) 0.1 mL of DMN diluted with glass-distilled water. When enzyme inhibitors are employed, the volume of PBS is reduced to 5.8 mL and 0.1 mL of the inhibitor dissolved in the appropriate solvent (metyrapone: 66 mM Tris-HCl, pH 7.5; α-naphthoflavone:dimethyl sulfoxide) is added. Controls lack S-15 and cofactors or the test compound or all three of these components.

The pH of the treatment medium is 7.5 when the test compound is added. After the tissue culture dishes have been placed in the incubator (5% CO2 in air) the pH drops to 7.0 and remains at this value for the duration of treatment.

Following a 2-hr incubation, the treatment medium is removed, the tissue culture dishes are washed once with 10 mL of phosphate-buffered saline and 10 mL of growth medium are added. The PBS and growth medium are warmed to room temperature prior to addition to avoid cold-stressing the cells. The cells to be used in the assessment of cytotoxicity are allowed to
grow for 7 to 9 days, at which time the colonies that form are stained (25 mL Giemsa stain, 30 mL absolute methanol, 945 mL glass-distilled water) and counted. Cytotoxicity is expressed as cloning efficiency relative to control. The cells to be used for the determination of mutation frequency are allowed an expression period equal to the time it takes for them to divide four times (16-cell stage, 48–80 hr after removal of the treatment medium, depending upon treatment toxicity). This is monitored with an inverted microscope. At this time, growth medium is removed and selective medium is added. The selective medium consists of growth medium containing 1 mM ouabain. The selective medium is changed 3 and 8 days after addition to remove dead cells and replenish nutrients. After 12 to 15 days of growth in the selective medium, the colonies which have formed are stained and counted. Mutation frequency is expressed on a per-survivor basis.

**Enzyme Assays**

**Ethoxyresorufin-O-deethylase (EROD).** EROD activity was assayed by a modification (21) of a previously described procedure (22). The amount of resorufin produced is quantified by comparison to a standard curve. The resorufin obtained from the supplier was recrystallized by dissolving in absolute ethanol, filtering (0.22 μm Millipore filter) and evaporating to dryness. The purified resorufin was redissolved in absolute ethanol for construction of the standard curve.

**Ethylmorphine-N-demethylase (EMND).** EMND activity was assayed as previously described (23). The amount of formaldehyde produced is determined by comparison to a standard curve constructed by carrying known amounts of formaldehyde through the incubation and assay procedures.

**Protein Assay**

The protein content of the S-15 fraction was measured by the Biuret method (24). Bovine serum albumin was employed as the standard.

**Results**

The cytotoxicity of DMN in the absence of the S-15 fraction is illustrated in Table 1. The cytotoxicity produced when 100 mM DMN is titrated with various, nontoxic (25) concentrations of S-15 protein is depicted in Figure 1. It is apparent that the observed cytotoxicity is biphasic in the presence of any of the five S-15 fractions.

To determine if DMN-induced cytotoxicity and mutagenicity are related, it is necessary to use a variety of treatments such that various numbers of cells survive each treatment and to measure the mutation frequency of each treatment in parallel with the cytotoxicity assessment. Toward that end, we chose to assay the cytotoxicity and mutagenicity of three different concentrations of DMN (10, 50 and 100 mM) in the presence of two different concentrations of S-15 protein (1.3 mg/mL and 3.5 mg/mL). This protocol was performed with S-15 fractions prepared from untreated rats as well as from rats pretreated with four different enzyme inducers [phenobarbital (PB) Aroclor 1254, β-naphthoflavone (BNF), MC]. After combining all of these data and plotting them on one graph (Fig. 2), it is apparent that the log of cloning efficiency is linearly related to mutation frequency (p < 0.01) when DMN is metabolically activated by the S-15 fraction.

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**Table 1. Cytotoxicity of dimethylnitrosamine in the absence of the S-15 fraction.**

| Dimethylnitrosamine, mM | Cloning efficiency, %<sup>a,b</sup> |
|------------------------|-----------------------------------|
| 0                      | 100 ± 2.9                          |
| 10                     | 96.8 ± 6.3                         |
| 50                     | 101.4 ± 1.4                        |
| 100                    | 87.3 ± 3.1                          |

<sup>a</sup>Expressed as a percentage of control.

<sup>b</sup>Mean ± SE of 12 replicates.
DMN is metabolically activated by an enzyme(s) termed DMN demethylase. We wished to determine whether DMN demethylase activity correlated with EROD activity or with EMND activity. The experimental approach for this series of experiments was identical with that previously described for studies on the metabolic activation of BP (25). Briefly, the experiments involve modulating the enzyme pattern contained in the hepatic postmitochondrial supernatant (S-15 fraction) in two manners: (1) the hepatic enzyme pattern is altered by pretreating experimental animals with enzyme inducers (phenobarbital, 3-methylcholanthrene, β-naphthoflavone or Aroclor 1254) and (2) selected enzymes in the S-15 fractions prepared from these animals are inhibited to various degrees in vitro with metyrapone (MET) or α-naphthoflavone (ANF). The EMND and EROD activities in a given S-15 fraction are determined and the ability of the preparation to produce mutagenicity and/or cytotoxicity in the presence of DMN is assessed. Correlations are made between the EMND or EROD activity in the S-15 fraction and the biological response of V79 cells in the presence of DMN. Since the concentration of S-15 protein in the reaction mixture markedly influences the cytotoxicity response to DMN (Fig. 1), all samples in this series of experiments contained equal amounts of S-15 protein (2.4 ± 0.1 mg/mL reaction mixture) while the DMN concentration always was 100 mM.

The EROD and EMND activity in S-15 fractions prepared from animals pretreated with selected enzyme inducers and the influence of α-naphthoflavone and metyrapone on these enzymes have been previously reported (25). The biological responses of V79 cells to 100 mM DMN when it is metabolized by these S-15 fractions was assessed and the data were combined and statistically analyzed as previously described (25). We
found that DMN-induced cytotoxicity or mutagenicity does not correlate significantly (p < 0.05) with EROD activity (Fig. 3) or with EMND activity (Fig. 4) in the S-15 fraction.

Discussion

The cytotoxicity curve which results when 100 mM DMN is titrated with various amounts of S-15 protein has a biphasic shape, regardless of whether the S-15 fraction is derived from an untreated rat or from one which has been pretreated with MC, BNF, Aroclor 1254 or PB (Fig. 1). Maximal toxicity is seen between 2.1 and 3.5 mg S-15 protein/mL reaction mixture, depending on the pretreatment used. When the concentration of S-15 protein is 3.5 mg/mL or greater, it is apparent that an increase in the amount of S-15 protein produces a decrease in observed cytotoxicity. In view of the widely accepted hypothesis that the metabolic activation of DMN to a cytotoxic compound is due to a single mixed-function oxidase catalyzed reaction, one would anticipate that the greater the amount of mixed-function oxidase (i.e., S-15 fraction) in the reaction mixture, the greater the rate of DMN activation and the greater the amount of DMN-induced cytotoxicity. Since DMN is less cytotoxic in the presence of large amounts of S-15 protein when compared to intermediate amounts, the excess protein is presumably acting as a nucleophilic trap for the DMN metabolite(s) responsible for cytotoxicity.

The data depicted in Figure 2 illustrate that cloning efficiency is linearly related to mutation frequency when DMN is metabolically activated by the S-15 fraction. This indicates either (A) the cytotoxic metabolite of DMN is identical with the mutagenic metabolite or (B) the cytotoxic and mutagenic metabolites of DMN are not identical, but are produced from DMN by microsomal enzymes in a constant ratio.

The metabolic activation of DMN is catalyzed by DMN demethylase (8). We wished to determine if DMN demethylase activity could be correlated with EROD activity, a reaction catalyzed by MC-inducible mixed function oxidases, or with EMND activity, a reaction catalyzed by PB-inducible mixed function oxidases. Very high concentrations of DMN (500–6700 mM) might denature and/or cause conformational changes in protein (26,27). However, there are numerous references in the literature which indicate that the DMN concentration employed in this study (≤100 mM) does not affect the activity of DMN demethylase (8,9,28).

The results of this study (Figs. 3 and 4) indicate that the cytotoxicity and mutagenicity of DMN neither correlate with EROD activity nor with EMND activity. There are several probable explanations for these results. The mixed-function oxidases whose activities are measured by the EROD and EMND assays are not the enzymes which metabolically activate DMN to toxic and mutagenic forms. Alternatively, the mixed-function oxidases whose activities are measured by EROD and EMND assays are responsible for the metabolic activation of DMN, but these enzymes even when substantially inhibited by MET or ANF, do not become rate-limiting. Also, the metabolic activation of DMN may be catalyzed by any one of several mixed-function oxidases. When one or more of these enzymes are inhibited by MET or ANF, other forms of cytochrome P-450 are able to compensate with little or no change in the rate of DMN activation.

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