Enhancement of Rat Liver Microsomal Metabolism of Azoxymethane to Methylazoxymethanol by Chronic Ethanol Administration: Similarity to the Microsomal Metabolism of N-Nitrosodimethylamine

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ABSTRACT

We compared the metabolism of azoxymethane (AOM) and of N-nitrosodimethylamine (NDMA) by liver microsomes obtained from male F344 rats pre-fed for 3 weeks either a control liquid diet or an isocaloric liquid diet containing ethanol at a concentration of 6.6% by volume. High-performance liquid chromatographic analysis of the products of the microsomal metabolism of AOM showed that methylazoxymethanol was the only primary metabolite. While the formation of small (less than 4%) of methylazoxymethanol quantities of methanol and formaldehyde could also be detected in this reaction, these products could be accounted for almost entirely by the spontaneous decomposition of methylazoxymethanol. With NDMA as the substrate in the incubation system, the formation of methanol, formaldehyde, methanol, and an additional, as yet unidentified metabolite was detected. Liver microsomes obtained from rats fed the ethanol-containing diet up to the time of sacrifice were 12-18 times more active in the metabolism of both AOM and NDMA than liver microsomes obtained from rats fed the control, ethanol-free diet for the same period. When rats fed the ethanol diet for 20.5 days were fed the control diet for 0.5 days and then sacrificed, only a 2- to 3-fold increase in the metabolism of both AOM and NDMA by liver microsomes was observed, indicating that cessation of ethanol intake results in a rapid decrease of the ethanol-induced metabolic enzymes. Hepatocytes isolated from ethanol-fed rats showed a significantly enhanced sensitivity to AOM as well as to NDMA-induced unscheduled DNA synthesis, indicating that the increased rate of microsomal metabolism induced by ethanol is associated with enhanced carcinogen activation in vitro. The metabolism of AOM and NDMA by liver microsomes was inhibited to similar extents by carbon monoxide, pyrazole, sodium azide, aminoacetonitrile, imidazole, and ethanol. In addition, both ethanol and NDMA were found to inhibit competitively the microsomal metabolism of AOM. These results suggest that AOM and NDMA are metabolized by very similar, indeed perhaps the same rat liver microsomal enzyme(s).

INTRODUCTION

AOM, a powerful colon, liver, and kidney carcinogen in rodents (1-4), is metabolically derived from 1,2-dimethylhydrazine via azomethane as an intermediate (1, 5, 6). AOM is metabolically activated by methyl group hydroxylation to MAM, a compound which can yield a DNA alkylating species, presumably the methylazidionion (1), either spontaneously (half-life, 11.6 h at pH 7 and 37°C, Ref. 7), or by enzyme-catalyzed reactions (7-9). Although the enzymatic hydroxylation of AOM to MAM appears to be a crucial step in the metabolic activation of both 1,2-dimethylhydrazine and AOM, up to now the reaction has been the subject of only relatively few studies (2, 5, 10-12). Structurally, AOM may be considered an isomer of the liver and kidney carcinogen, NDMA, which is also activated by methyl group hydroxylation (13). Indeed, on the basis of theoretical considerations, Miller (14) and also Druckrey (1) independently predicted similarities between the metabolic activation of AOM and NDMA. However, this concept has recently been questioned (4, 12). In contrast to the relative lack of attention given to studies of enzymes involved in the activation of AOM, much more work has been performed on the characterization of enzymes catalyzing the activation of NDMA, and a great deal of information has been obtained on the metabolism of NDMA by microsomal fractions, purified reconstituted microsomal components (15-18), as well as on the effects of inducers (19-22) and inhibitors (23-29) of NDMA metabolism.

Because of the strong association found among chronic alcohol consumption, smoking, and development of cancer of the head and neck (reviewed in Refs. 30 and 31), the effects of alcohol as a modifier of the metabolism of NDMA and other carcinogenic N-nitrosamines have received special attention (20, 21, 32-35). A positive correlation between the consumption of alcoholic beverages and the development of human colorectal cancer has also been reported (36, 37), and several attempts have been made to test this correlation under controlled laboratory conditions using the colon carcinogens 1,2-dimethylhydrazine or AOM in the rat model. However, the results of these studies have been conflicting (38-40). With the assumption that some of these apparent discrepancies might be resolved if the effects of ethanol on the enzymatic steps in the activation of these colon carcinogens were better understood, we began studies on relationships between chronic and acute ethanol administration and the metabolic activation of AOM in F344 rats (41). During that work, we noted several striking similarities in the metabolism of AOM and of NDMA by liver microsomes, including the ability of chronic ethanol feeding to greatly enhance the metabolism of both carcinogens in vitro. In the present report we further examine and document the similarities observed. Our results suggest strongly that the activation of AOM and of NDMA by rat liver microsomes are carried out by very similar, indeed perhaps the same, enzymes.

MATERIALS AND METHODS

Chemicals. AOM was purchased from Ash Stevens Inc., Detroit, MI. NADP+, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, metyrapone, imidazole, and NDMA were obtained from Sigma Chemical Co., St. Louis, MO. Aminoacetonitrile and methylazoxymethyl acetate were obtained from Aldrich Chemical Co., Milwaukee, WI. DNFB was obtained from ICN Pharmaceuticals, Plainview, NY, and...
As well as to accelerate the elution of basic (cationic) metabolites by ion exchange with 0.2 M ammonium phosphate buffer, pH 3.1, at a flow rate of 0.5 ml/min for the first 24 min, and at 1 ml/min thereafter. Absorbance was measured at 215 nm, indicating spontaneous decomposition, also characteristic of MAM. The HPLC elution volume (13.5 ml), which was likewise identical to that of the standard, with a characteristic absorbance maximum at 215.5 nm. In addition, incubation of this metabolite at 37°C and pH 3.1 in a sealed silica cuvette led to a time-dependent loss of absorbance at 215 nm, indicating spontaneous decomposition, also characteristic of MAM. The minor peaks appearing at 9.5 and 10.5 ml were identified as spontaneous decomposition, also characteristic of MAM.

**Identification of Methylamine as an Urinary Metabolite of NDMA.**

Methods used were similar to those described previously (10) for the identification of methylamine as a urinary metabolite of AOM in pyrazole-pretreated rats. DNP derivatives of methylamine, dimethylamine, 1,1-dimethylhydrazine, and monomethylhydrazine, to serve as chromatographic standards, were prepared by adding 50 μL DNF to 100 mg of each compound in 2.0 ml of 37.5% (w/v) solution of Na2SO4: 10 H2O. After stirring for 1 h at 25°C, 1 ml of a saturated solution of t-glutamic acid was added to deplete excess reagent, and stirring was continued for 30 min. The mixtures were exhaustively extracted with ethyl acetate. The extracts were taken to dryness under a stream of N2.

The residues were dissolved in methanol and submitted to TLC using 250 μm silica gel plates eluted with benzene. Rf values of the derivatives were: DNP-methylamine, 0.40; DNP-dimethylamine, 0.45; DNP-monomethylhydrazine, 0.18; DNP-1,1-dimethylhydrazine, 0.53. Bands corresponding to purified DNP derivatives were scraped from the plate, and derivatives were eluted from the silica gel with methanol. For HPLC, the methanol extracts were taken to dryness under N2, redissolved in n-hexane:ethanol (8:2, v/v) and aliquots were applied to two Waters 

**Preparation of Liver Microsomes.** Livers were excised and homogenized in three weight volumes of ice-cold 0.25 M sucrose, 0.01 M potassium phosphate buffer, pH 7.5. Homogenates were centrifuged at 9,000 × g for 20 min and microsomes were obtained by centrifuging the resulting supernatant at 100,000 × g for 1 h. Microsomes were washed by suspending the pellet in the buffer used for homogenization and centrifuging at 100,000 × g for 1 h. The washed microsomes were resuspended in three weight volumes of 0.1 M potassium phosphate buffer, pH 7.0, and divided into several portions which were kept at −70°C until assayed.

**Incubation Conditions and HPLC Analysis of Metabolites.** Incubation mixtures consisted of 3.5 mM glucose-6-phosphate, 5 units of glucose-6-phosphate dehydrogenase, 1.5 mM NADP+, 3.5 mM MgCl2, 0.1 M phosphate buffer (pH 7.0), labeled substrate, and approximately 1 mg microsomal protein in a total volume of 0.5 ml. For quantitative comparisons of enzymatic activity of liver microsomes from animals on the various liquid diets, substrate concentrations used were 3 mM [14C]AOM (specific activity, 0.4 mCi/mmol) or 5 mM [14C]NDMA (specific activity, 0.4 mCi/mmol). In experiments examining the effects of various enzyme modifiers on the metabolism of the carcinogens, the concentration of the substrates as well as of the modifiers in the incubation mixtures was 1 mM each.

After incubation at 37°C for 15 min in a shaker bath, the reaction vessels were quickly chilled to 0°C. The suspensions were deproteinized by centrifugal ultrafiltration (600 × g, 30–60 min, 0–4°C) using Centrifree Micropartition system tubes (Amicon, Danvers, MA). Ultrafiltrates were submitted to HPLC using two Whatman ODS-3 (0.46 × 25-cm) columns in series, preceded by a 0.7 × 5-cm column packed with Aminex A-29 anion-exchange resin (Bio-Rad Labs., Rockville Centre, NY) in the phosphate form. The column system was eluted with 0.2 M ammonium phosphate buffer, pH 3.1, at a flow rate of 0.5 ml/min for the first 28 min, and at 1 ml/min thereafter. Absorbance was monitored at 215 nm and fractions were collected at 1-min intervals for determination of radioactivity. The anion-exchange precolumn served to retard possible acidic (anionic) metabolites by ion exchange as well as to accelerate the elution of basic (cationic) metabolites by ion exclusion. The application of this HPLC system to the identification of formic acid as an in vitro metabolite of MAM has been described elsewhere (9). For rapid routine assays of microsomal NDMA metabolism, we also used the method of Nash (43), as modified by Tu and Yang (44), to quantitate formic acid.

**RESULTS**

Microsomal Metabolism of AOM and NDMA: Stimulation by Ethanol Feeding. Incubation of 14C-labeled AOM with an NADPH-generating system and liver microsomes from rats fed for 21 days either control liquid diet or the isocaloric ethanol diet resulted in the formation of one major and two minor labeled species, as shown by the HPLC profiles in Fig. 1. The major metabolite was identified as MAM on the basis of its retention times and absorbance characteristics. The minor peaks appearing at 9.5 and 10.5 ml were identified as formaldehyde and methanol, respectively, on the basis of their elution volumes. All three products were observed to form in incubation systems using ethanol-induced or control liver microsomes.
of 20 ml. Although this peak eluted at a volume similar to that of formic acid, a reported metabolite of NDMA (47, 49), the elution volumes did not coincide and the identity of this product remains to be established. In terms of total products formed, methanyle was represented 6.2 ± 0.8% and 9.8 ± 4.8% for ethanol-induced and control microsomes, respectively; the analogous percentages for formaldehyde were 45.3 ± 3.3% and 42.8 ± 6.6%; for methanol these were 40.0 ± 3.1% and 36.4 ± 7.4%; and for the unknown product, 8.6 ± 5.5% and 8.6 ± 1.4% (means ± SD obtained from 11–12 determinations). These estimates are based on the assumption that the corresponding HPLC peaks are homogeneous.

The time courses of total product formation from AOM and NDMA, using liver microsomes from rats fed either the ethanol diet for 21 days (group I), the ethanol diet for 20.5 days followed by the control liquid diet for 0.5 day (group II) or the control diet for 21 days (group III) are shown in Fig. 3. The results indicate that the metabolism of AOM (present in the incubation mixtures at 3 mM) to MAM was linear for at least 15 min with liver microsomes from all three groups of animals. In the case of NDMA (present in the incubation mixtures at 5 mM) metabolism was linear for at least 10 min. It is evident from these data as well as from Figs. 1 and 2 that liver microsomes from rats fed the ethanol diet up to the time of sacrifice were 12–18 times more active in metabolizing AOM and NDMA than liver microsomes from rats fed the control diet. In Fig. 3, the data for AOM and NDMA were each obtained using liver microsomes from different rats in separate feeding experiments carried out at different times. In other assays, when the same induced or control liver microsomes were used to examine the metabolism of both AOM and NDMA, very similar levels of activity for both reactions were observed. Thus, with liver microsomes from rats on control liquid diet, AOM and NDMA, each at a concentration of 1 mM in the incubation mixtures, were metabolized at the rates of 0.80 ± 0.13 nmol/min/mg protein (N = 5) and 0.70 ± 0.10 nmol/min/mg (N = 5), respectively. Using liver microsomes from rats on the ethanol diet, AOM (1 mM) and NDMA (1 mM) were metabolized at the rates of 14.3 ± 0.95 nmol/min/mg (N = 5) and 13.9 ± 1.8...
(N = 7) nmol/min/mg, respectively. It is significant that, as illustrated in Fig. 3, liver microsomes isolated from rats which were switched from the ethanol to the control liquid diet 12 h prior to sacrifice were only about three times more active in both AOM and NDMA metabolism than liver microsomes isolated from rats fed the control diet. These results indicate that chronic ethanol feeding of rats causes a parallel increase in the ability of liver microsomes to metabolize both carcinogens, and that interruption of ethanol intake results in similar rapid losses in metabolic activity.

Effects of Ethanol Feeding on AOM- and NDMA-induced UDS in Isolated Hepatocytes. Enhancement of NDMA metabolism by rat liver microsomes following chronic ethanol administration has been accompanied by increased microsomal activation of NDMA to a mutagen for S. typhimurium and Chinese hamster cells (20, 50, 51), and in a potentiation of the UDS response to NDMA by isolated rat hepatocytes (52). We have previously reported that liver microsomes are active in MAM metabolism (8, 9), and that such activity can also be stimulated many fold by chronic ethanol feeding (41). The enhancement of microsomal metabolism of AOM to MAM following chronic ethanol administration as documented in Figs. 1 and 3 therefore suggested that this process should result in a concomitant increase in the overall conversion of AOM to a DNA-reactive species. To test this possibility, AOM, with NDMA as a positive control, was assayed at various concentrations for induction of UDS (45, 46) in hepatocytes isolated from rats in group I (ethanol diet, 21 days) and group III (control liquid diet, 21 days). The results, shown in Table 1, indicate that hepatocytes from rats fed the ethanol diet, respond with UDS to concentrations of either AOM and NDMA which are 10 or more-fold lower than those producing roughly the same degree of UDS in hepatocytes isolated from rats fed the control liquid diet. Thus, the stimulation of AOM metabolism by ethanol feeding results in increased activation of the compound to a genotoxic species in vitro, and this effect is again parallel to that observed with NDMA.

Effects of Enzyme Modifiers on the Metabolism of AOM and NDMA by Rat Liver Microsomes. Carbon monoxide (15), pyrazole (24, 25, 27, 29), sodium azide (25), aminoacetoniitrile (23, 28, 29), imidazole (27), and ethanol (21) have been reported as inhibitors of the in vitro metabolism of NDMA. Pyrazole has also been described to inhibit AOM metabolism in vitro as well as in vivo (10), and both pyrazole and aminoacetoniitrile were shown to inhibit the mutagenicity of AOM for S. typhi-

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<th>Table 1</th>
<th>Unscheduled DNA synthesis induced by NDMA and AOM in hepatocytes isolated from rats fed control and ethanol liquid diets for 21 days</th>
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<td>Each assay was performed using hepatocytes from one rat.</td>
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<td><strong>Grains per nucleus ± SD</strong></td>
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* Male F344 rats maintained on isocaloric ethanol (6.6% by volume) for 21.0 days prior to isolation of hepatocytes. |
* Male F344 rats pair fed control liquid diet for 21.0 days prior to isolation of hepatocytes. |
* ND, not determined. |
* ++++, number of grains too great for quantitation (≥200). |

Fig. 4. Comparative effects of enzyme modifiers on the metabolism of 14C-labeled AOM and NDMA by liver microsomes from rats fed liquid control or ethanol diets for 21 days. Substrate and modifier concentrations were 1 mM. In the case of carbon monoxide, CO2 (8:2, v/v) was bubbled through the incubation mixtures for 10 min at 0°C prior to incubation, and incubation was carried out under a CO2 atmosphere of the same composition. For control (less modifier) activity levels, see "Results" section. In the case of AOM: values, average of two separate determinations; bars, range. In the case of NDMA: values, mean of three separate determinations each using liver microsomes from one animal; bars, ± SD. |

Fig. 5. Dixon plots for ethanol inhibition of 14C-labeled AOM metabolism by liver microsomes from rats fed control liquid diet (4) or ethanol diet (8) for 21 days. Ordinates, reciprocal of the rate of formation of labeled MAM as determined by HPLC. For control and ethanol induced microsomes, the K values for ethanol were determined as 0.18 and 0.63 mm, respectively. |
ethanol-induced liver microsomes, respectively. Competitive inhibition of rat liver microsomal metabolism of NDMA to formaldehyde by ethanol has been described previously (21).

**DISCUSSION**

The present data indicate that liver microsomes isolated from rats maintained for a period of 21 days prior to sacrifice on a liquid diet containing 6.6% ethanol are 12–18 times more active in the metabolism of AOM and NDMA than liver microsomes from rats maintained for the same period of an isocaloric ethanol-free control liquid diet. This degree of induction of microsomal enzyme activity in response to ethanol administration is much higher than that reported by Garro et al. (20), who found a 1.5–1.7-fold enhancement of NDMA demethylease activity in liver microsomes isolated from male Sprague Dawley rats fed a liquid diet containing ethanol as 36% of total calories for 4 weeks. The level of induction noted by us is also higher than that reported by Yoo and Yang (51), who found a 4.5-fold increase in liver microsomal NDMA demethylease activity in young male Sprague Dawley rats after 3 days of treatment with 15% ethanol in the drinking water. The apparent low degree of induction found by Garro et al. (20) may probably be accounted for by our finding that cessation of ethanol intake is followed by a rapid loss of the ability of liver microsomes to metabolize both NDMA and AOM. This is evident from the data in Fig. 3, which show that liver microsomes from rats fed ethanol for 20.5 days and then fed the control diet for 0.5 days were only three times more active than were liver microsomes from rats fed the control diet. In the case of the experiments performed by Garro et al. (20), the rats were taken off the ethanol diet overnight prior to sacrifice, and this then would be expected to lead to a significant loss of NDMA demethylease activity. Similar rapid decreases in other ethanol-induced microsomal enzyme activities, including aniline hydroxylase, and 7-ethoxycoumarin-O-deethylease, due to cessation of ethanol intake have been previously noted by Hetu and Joly (55). Whether a similar situation occurs in humans is unknown, but the apparent short half-life of ethanol-induced AOM- and NDMA-microsomal metabolizing enzymes in the rat may need to be taken into account in laboratory experiments intended to model the effects of alcohol consumption on carcinojen activation in the human. The differences between our results and those of Yoo and Yang (51) may be explained by differences in the strains and ages of animals used and by differences in the protocols of ethanol administration.

Previously, using HPLC with UV detection, our laboratory has shown that MAM was produced during the incubation of AOM with rat liver microsomes plus an NADPH-generating system (5). In the present work, using AOM labeled with carbon-14 in both methyl groups, we have demonstrated that MAM is the only primary metabolite of microsomal metabolism of AOM detectable by the HPLC system used. The small amounts of formaldehyde and methanol produced in this reaction are less than 4% of the MAM formed. Within experimental error, the production of this much methanol and formaldehyde may be accounted for almost entirely by the spontaneous decomposition of MAM. Other workers (2, 11) have used the Nash reaction (43) to measure the microsomal metabolism of AOM by a presumptive “AOM demethylase.” However, our results indicate that rat liver microsomes do not directly catalyze the demethylation of AOM to formaldehyde, but rather the hydroxylation of AOM to MAM. It is likely that the formaldehyde measured by others (2, 11) may in fact have been derived from the decomposition of the unstable MAM metabolite during the employment of the Nash reaction.

The results reported here demonstrate strong similarities between the metabolism of AOM and NDMA by rat liver microsomes. Both activities are induced to approximately the same degree by ethanol, and both activities decline to about the same degree upon cessation of ethanol intake. The stimulation of carcinogen metabolism is accompanied by a greater sensitivity of hepatocytes isolated from ethanol-fed rats to AOM- as well as to NDMA-induced unscheduled DNA synthesis, suggesting that the increases in metabolism observed with microsomes in vitro could be a reflection of increased rates of AOM and NDMA activation in vivo. However, our results also show that ethanol is a competitive inhibitor of AOM hydroxylation (Fig. 5). Thus, the possibility exists that even though ethanol feeding causes a many-fold induction of enzymes activating these carcinogens, the presence of ethanol in the animal may have a net effect of inhibiting its metabolism to levels below that of nonethanol treated controls. Indeed, in a separate work (56), we show that the in vivo metabolism of 14C-labeled AOM in F344 rats maintained on an ethanol liquid diet up to the time of carcinogen administration (analogous to group I in the present study) is significantly inhibited for at least 5 h in comparison to metabolism in rats maintained on control liquid diet (as in group III, present study). On the other hand, we found (56) that the initial rate of metabolism of labeled AOM was significantly enhanced in rats which had been fed the ethanol diet for 20.5 days and then fed the control liquid diet for 12 h prior to carcinogen administration (as in group II, present study). It is evident that the effects of ethanol on the rates of metabolic activation of AOM in vivo can be quite complex, and will be strongly dependent on the amount, duration, as well as the timing of administration of both the ethanol as well as the carcinogen (39, 41, 56). It is possible that variations in these factors may have contributed to the apparent discrepancies regarding the effects of ethanol on colorectal carcinogenesis in animal models (38–40). A discussion of similar opposing effects of ethanol on the metabolic activation of NDMA has been presented by Peng et al. (21).

In addition to the similarities in the induction and decrease of liver microsomal enzymes metabolizing AOM and NDMA, our results show further similarities between the metabolism of the two carcinogens with respect to the effects of various enzyme modifiers. From the data in Fig. 4, it is evident that carbon monoxide, pyrazole, sodium azide, aminooctonitrile, and imidazole inhibit the metabolism of both AOM and NDMA to approximately the same extents regardless of whether control
or ethanol-induced microsomes are used in the assay. It is of interest that NDMA inhibits the metabolism of AOM and, conversely, that AOM inhibits the metabolism of NDMA. With respect to AOM metabolism, NDMA is a powerful competitive inhibitor (Fig. 6), indicating binding of both carcinogens to the same enzyme site. The differences in the apparent $K_i$ values obtained using liver microsomes from ethanol-treated and control rats may indicate that the ethanol-induced and basal forms of the AOM metabolizing enzymes are different; however, this possibility can be tested unambiguously only when purified forms of the enzymes become available.

Considering the results presented here, we suggest that AOM and NDMA are metabolized by very similar, indeed, perhaps the same rat liver microsomal enzyme(s). Curiously, Lijinsky et al. (4, 12) came to the opposite conclusion, based to a great extent on the results of their experiments which seemed to show that rat liver microsomes, while active in NDMA metabolism, did not metabolize AOM to any significant extent (12). In contrast, our results indicate that F344 rat liver microsomes metabolize AOM and NDMA almost equally well. A probable cause for the difference between our results and those of Lijinsky et al. (12) may be their use of an HPLC system for the analysis of AOM metabolites that was incapable of resolving MAM from AOM. Examination of Fig. 4 in Ref. 12 shows that 20% acetonitrile-water was used to elute a single Zorbax ODS reverse-phase HPLC column for the analysis of products of AOM metabolism. In our hands, we find this eluant too non-polar for resolving AOM and MAM on reverse-phase columns. Because AOM substrate and MAM product will elute in a single peak under these conditions, it is possible to come to the erroneous conclusion that metabolism did not occur. For efficient separation of AOM and MAM, the use of acetonitrile or methanol at concentrations greater than about 1 or 2% in the eluant should be avoided.

In summary, our data show that both AOM, a colon, kidney and liver carcinogen in rats, and NDMA, a liver and kidney carcinogen, are metabolically activated by very similar rat liver microsomal enzymes which are induced as well as inhibited by ethanol. Further studies with purified microsomal components will be necessary to decide whether these enzymes are or are not identical.

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