Organ Specificity in the Microsomal Activation and Toxicity of N-Nitrosomethylbenzylamine in Various Species

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ABSTRACT

The microsomal metabolism of the rat esophageal carcinogen N-nitrosomethylbenzylamine (NMBZA) at the methylene carbon atom to yield benzaldehyde was studied in various organs of a number of species to determine the role of metabolic activation in the carcinogenicity or toxicity of the nitrosamine. In the Sprague-Dawley rat, NMBZA was metabolized by microsomes from liver, lung, and esophageal mucosa. In the F344 rat and rabbit, metabolic activity was present in both liver and esophageal mucosa, the only tissues studied in these species. In contrast, in the Syrian hamster and BALB/cByJ mouse, NMBZA debenzylation was undetectable in the esophagus but occurred at relatively high rates in liver, lung, and kidney. The forestomach mucosa exhibited undetectable levels of activity in the Sprague-Dawley rat and BALB/cByJ mouse, although in the hamster, it was present at a very low level. Administration of a dose of NMBZA acutely toxic to the rat (18 mg/kg i.p.) resulted in significant cellular damage only to the rat esophageal mucosa, no other tissues examined in the rat, hamster, or mouse being affected. These observations, together with the available data on carcinogenicity of the nitrosamine in the rat and rabbit, suggest that in the esophagus, at least, metabolic activation of NMBZA is necessary to elicit its toxic and/or carcinogenic effect. However, NMBZA is also metabolized at a high rate in the liver of all species but is not toxic or carcinogenic in this tissue, suggesting that other factors besides metabolic activation must be involved in the resistance of hepatocytes to the effects of the nitrosamine. Microsomes prepared from human esophageal mucosa from six patients metabolized NMBZA at rates that were either undetectable or approximately 70 times lower than in the Sprague-Dawley rat.

INTRODUCTION

Nitrosamines produce tumors in a wide variety of tissues in experimental animals (21). The biological basis for this tissue specificity, however, is poorly understood. NMBZA, one of the most potent of the carcinogenic nitrosamines, is highly selective in inducing tumors of the esophagus and pharynx in the rat, independent of its route of administration (7, 30). Esophageal tumors have also been induced in rabbits by p.o. treatment with sodium nitrite and N-methylbenzylamine (13). In NMRI mice, the route of administration influences the organ specificity of NMBZA. Administration p.o. causes tumors of the esophagus and forestomach, whereas forestomach carcinomas and lung adenomas are induced by s.c. injection (27).

A number of studies have shown that the organ specificity of NMBZA is not caused by distribution effects (12, 16); tissue-specific metabolic activation, however, probably plays an important role. We have shown previously that microsomes prepared from rat esophageal mucosa metabolize NMBZA at a high rate to yield almost exclusively benzaldehyde and a methylating agent in a cytochrome P-450-dependent reaction (NMBZA debenzylation) (17). Furthermore, studies of DNA methylation in rats and mice have indicated that the capacity of tissues to activate NMBZA may influence their susceptibility to tumor induction (11, 14).

To investigate further the role of metabolic activation in the organ-specific toxicity and carcinogenicity of NMBZA, in the present study we have examined the NMBZA debenzylation activity of microsomes prepared from the esophagi of the Sprague-Dawley rat, F344 rat, New Zealand White rabbit, BALB/cByJ mouse (NMRI mice are unavailable in North America), and Syrian golden hamster. The results have been compared to those obtained with microsomes prepared from several other tissues. The target organs for NMBZA in the Syrian hamster and BALB/cByJ mouse are not known, although induction of oral cavity tumors by application of NMBZA to the buccal pouch of Syrian hamsters has been briefly reported recently (29). We have, therefore, examined the acute toxicity of NMBZA in these species and compared it to that in the Sprague-Dawley rat to determine whether there is a relationship between metabolism and biological effects of the nitrosamine in various organs. In order to evaluate the susceptibility of the human esophagus to possible carcinogenic effects of NMBZA, the metabolic activity of microsomes prepared from a number of surgical specimens has also been measured.

MATERIALS AND METHODS

Chemicals. NMBZA (b.p. 60-62°C/0.01 mm), synthesized by the method of Druckrey et al. (7), was 99% pure as determined by gas chromatography and high-performance liquid chromatography. NADP (sodium), glucose 6-phosphate (sodium), glucose-6-phosphate dehydrogenase (type XV), and bovine serum albumin (Fraction V) were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were from Fisher Scientific Co., Fairfawn, NJ.

Animals. Male Sprague-Dawley rats (21 to 23 days old), male Syrian golden hamsters (21 to 23 days old), and male F344 rats (28 to 30 days old) were purchased from Charles River, Inc., Laprairie, Quebec, Canada. Male BALB/cByJ mice (21 to 28 days old) were purchased from The Jackson Laboratory, Bar Harbor, ME. These animals were maintained on a Teklad 6% fat rat-mouse diet ad libitum. Male New Zealand White rabbits (9 to 10 weeks old), purchased from Riemens Fur Ranches, Ltd.,...
Enzyme Preparation. After 5 to 10 days of acclimatization, animals were sacrificed by CO₂ asphyxiation. Microsomes from the liver and esophageal mucosa of all species were prepared as described previously (17). Lung and kidney were homogenized in 3 volumes of buffer (1.15% KCl-50 mM Tris-HCl, pH 7.4) with 30 passes of a motor-driven Potter-Elvehjem homogenizer with Teflon pestle (kidney). Forestomachs were slit open along their greater curvatures, cleared of contents, and rinsed in homogenizing buffer. The mucosa was scraped off with a scalpel and then homogenized in the same way as esophageal mucosa. Microsomes from all tissues were prepared as described for liver and esophageal mucosa (17). For each microsomal preparation, tissues were pooled from the following number of animals: for liver, 3 animals; for lung and kidney, 3 rats, 10 hamsters, or 10 mice; for forestomach and esophageal mucosa, 10 rats, 20 hamsters, or 80 mice. In the case of the rabbit, both liver and esophageal mucosa were pooled from 2 animals.

NMBZA Metabolism. Incubations were carried out at 37° for 20 min (or 60 min for human esophageal microsomes) in the presence of 5 mM NMBZA, approximately 0.5 mg of microsomal protein, an NADPH-generating system, and semicarbazide exactly as described previously (17). Benzaldehyde semicarbazone, benzyl alcohol, and benzoic acid were determined by high-performance liquid chromatography (17). Detection limits (nmol/min/mg protein) were: benzaldehyde, 0.005; benzyl alcohol, 0.10; and benzoic acid, 0.03.

Acute Toxicity Studies. Sprague-Dawley rats, mice, and hamsters were given a single i.p. dose of NMBZA (18 mg/kg body weight) in dimethyl sulfoxide (0.1 ml/100 g body weight). Control animals were given injections i.p. of dimethyl sulfoxide (0.1 ml/100 g body weight). Mice were also treated p.o. with NMBZA (18 mg/kg) by mixing the nitrosamine in drinking water. The controls in this instance were provided with drinking water without any additions. Each control or treated group consisted of 3 animals. Throughout this study, animals were provided with food and water ad libitum. After a 48-hr period of observation, the animals were sacrificed. Liver, esophagus, kidney, forestomach, and lung were removed, examined grossly, and then fixed in 10% neutral buffered formalin prior to being processed for light microscopy.

Other Analyses. Protein was estimated by the method of Lowry et al. as described by Munro and Fleck (23) with bovine serum albumin as standard. Statistical analyses were carried out by using 2-tailed Student’s t tests.

RESULTS

Our previous studies have shown that the major product of the metabolism of NMBZA in both the liver and esophagus of the Sprague-Dawley rat is benzaldehyde (17). This product is formed by oxidation of NMBZA at the methylene carbon atom to form the α-hydroxynitrosamine followed by spontaneous fragmentation of this intermediate; a methylyating agent is the other product of this fragmentation. Benzyl alcohol is also formed mainly by enzymatic reduction of benzaldehyde, despite the presence of semicarbazide in the incubation mixture. Oxidation of benzaldehyde to benzoic acid does not take place with microsomes from liver or esophagus (17). In the liver, oxidation of NMBZA at the methyl carbon to yield formaldehyde and a benzoylating agent is 10 times slower than oxidation at the benzyl moiety; in the esophagus, formaldehyde formation from NMBZA is more than 100 times slower than benzaldehyde formation (17). Furthermore, benzaldehyde formation from either rat liver and esophageal DNA is undetectable and probably plays no role in carcinogenesis by NMBZA (10). For the purpose of this study, therefore, formaldehyde formation was not measured. It is known that rat hepatic NDMA-demethylase exists as at least 2 isozymes with different kinetic and regulatory properties (1). Whether similar isozymes exist for NMBZA-debenzylation remains to be determined. The debenzylation activities are measured here at an NMBZA concentration of 5 mM, which corresponds to a saturation level of NDMA for NDMA-Demethylase I (1).

In the present studies, NMBZA was metabolized to benzyaldehyde and benzy alcohol by microsomes from the liver and esophageal mucosa of the Sprague-Dawley rat (Chart 1a) at rates similar to those observed previously (17). A significant level of NMBZA-debenzylation activity [0.155 ± 0.025 (S.E.) nmol benzaldehyde/min/mg protein] was also detected in microsomes prepared from whole lung of the Sprague-Dawley rat, although this activity was about one-fourth of that in the esophageal mucosa. Both the hepatic and esophageal metabolic profiles in the F344 rat (Chart 1b) were the same as in the Sprague-Dawley rat, although there were interstrain differences in rates of metabolism in both tissues.

With rabbit liver microsomes (Chart 1c), benzaldehyde was...
produced at a similar rate to that in the F344 rat, although benzyl alcohol formation was very much reduced. With esophageal microsomes from the rabbit, NMBZA was metabolized at about one-fourth of the rate of that in the Sprague-Dawley rat. Unlike the rat, however, the rabbit produced benzoic acid at a significant rate with both hepatic and esophageal microsomes.

In both the Syrian hamster and the BALB/cByJ mouse (Chart 2), high levels of NMBZA-debenzylase activity were observed with liver microsomes with formation of benzaldehyde, benzyl alcohol, and benzoic acid. In contrast to the rat and rabbit, NMBZA was not metabolized by esophageal microsomes from either hamster or mouse. Lung and kidney microsomes from both hamster and mouse, however, metabolized NMBZA at rates significantly higher than microsomes from these tissues in the rat. A low level of NMBZA metabolism to both benzaldehyde and benzoic acid was also observed in microsomes from the forestomach mucosa of the hamster but not in the mouse (Chart 2).

In order to relate our metabolism studies to the biological effects of NMBZA, we have compared gross and histopathological changes over a 48-hr observation period in various organs of hamsters, mice, and Sprague-Dawley rats following i.p. administration of the nitrosamine at a dose of 18 mg/kg body weight, which is the p.o. 50% lethal dose in the BD rat (6). No gross abnormalities were observed at any stage in either the control rats or control and nitrosamine-treated mice and hamsters. After 24 hr, however, the treated rats began to appear cachectic, and one of the 3 rats was found dead at 48 hr. Macroscopic examination of various organs 48 hr after treatment revealed no significant changes except for the rat esophagus, in which the mucosa in certain places appeared to be loosely attached to the submucosal and muscle layers.

Histopathological changes in the treated rat esophagus included acanthosis of the squamous epithelium with architectural disorganization of epithelial basal layers and mononuclear inflammatory infiltration of mild degree (Fig. 2; Fig. 1 shows untreated rat esophagus for comparative purposes). Plasma cells and lymphocytes were mainly perivascular in distribution throughout the esophageal wall (Fig. 3). In addition, some of them were present in the submucosa, underneath the basement membrane with no relation to the vascular channels (Fig. 2). These changes are similar to the preneoplastic lesions that have been observed 3 months after continuous treatment of male albino rats with N-methyl-N-nitrosourea (24). In our study, there was no evidence of erosion, ulceration, or squamous cell necrosis in the basal layers ("colloid bodies"). However, focal exocytosis was present, and apoptotic cells were seen in the midportion of the epithelium (Fig. 2). Erosions in areas of the esophagus on the first day after administration of NMBZA to male Donryu rats described by Izuka et al. (12) can perhaps be accounted for by the extremely high dose of NMBZA (83 mg/kg) used in their study. In the hamster and mouse, light microscopy showed no pathological abnormalities in either the liver, lung, esophagus, forestomach, or kidney of treated animals. Whereas the esophagi from mice treated i.p. with NMBZA were similar to untreated controls (Fig. 4), the same dose of NMBZA administered in the drinking water produced focal inflammatory infiltration in the submucosa (Fig. 5) after 48 hr. The infiltrate was composed of polymorphonuclear cells and lymphocytes. The overlying squamous epithelium showed disorganization of the basal layers. This suggests that, by the p.o. route, NMBZA has a slight toxic effect in the esophagus. The pathological change in the mouse esophagus, however, was nowhere near as extensive as in the rat esophagus.

The capacity of the human esophageal mucosa to activate NMBZA was studied in microsomes prepared from disease-free tissue obtained from 6 patients undergoing surgery for either esophageal cancer or chronic penetrating ulcer. The data for individual patients are presented in Table 1. Compared to rat esophagus, an extremely low level (72-fold lower) of benzaldehyde formation was detected when NMBZA was incubated with microsomes from human esophageal mucosa. The rate of benzaldehyde formation in the human samples ranged from undetectable to 0.015 nmol/min/mg protein. Further metabolism of benzaldehyde to benzyl alcohol or benzoic acid was not detected in human esophageal microsomes.

**DISCUSSION**

The rates of microsomal metabolism of NMBZA at the methylene carbon we have observed in various organs of the Sprague-Dawley rat are similar to the results of Hodgson et al. (11) for...
male Wistar rats treated with NMBZA, in which the highest level of DNA methylation, after liver and esophagus, occurred in the lung. These investigators also detected low levels of DNA methylation in forestomach and kidney in Wistar rats, and the presence of NMBZA-debenzylase activity in the forestomach of female SIV 50 rats has been reported by Schweinsberg and Kouros (28). We were unable to detect any metabolism of NMBZA in either forestomach or kidney of Sprague-Dawley rats, however, suggesting strain and/or sex differences.

Following i.p. administration of NMBZA in female NMRI mice, the highest levels of DNA methylation were observed in liver followed by lung, forestomach, esophagus, and kidney (14). Schweinsberg and Kouros (28) also found detectable levels of NMBZA-debenzylase activity in the forestomach of female NMRI mice. While our metabolic data for liver, lung, and kidney from BALB/cByJ mice parallel the relative extents of DNA methylation observed in the same tissues in NMRI mice, the undetectable levels of enzyme activity in forestomach and esophageal mucosa in our studies suggest sex and/or strain differences. Metabolic activation of NMBZA has not been studied previously in the Syrian hamster, although nitrosodimethylamine and nitrosodiethylamine are metabolized by liver, kidney, respiratory tract, esophagus, and small intestine of this species (22).

Although the liver in all species exhibited the highest level of metabolic activity for NMBZA, in the Sprague-Dawley rat, the F344 rat, and rabbit, no liver tumors have been observed (7, 8, 13, 30). In the BALB/cByJ mouse and the Syrian hamster, in which the carcinogenicity of NMBZA is unknown, our studies have indicated no evidence of toxicity in the liver. Furthermore, in the rat at least, the liver is one of the predominant sites for uptake of NMBZA (12, 16). In the rat, NMRI mouse, and the gerbil, high levels of the promutagenic base O\(^2\)-methylguanine are initially formed in the liver after NMBZA treatment, although no liver tumors have been detected in these species (3, 11, 14, 32). These observations suggest that, while metabolic activation may be the necessary first step in toxigenesis or carcinogenesis by nitrosamines, it is not sufficient to elicit these effects. The rate of DNA repair and replication may be critical for the fixation and expression of damage induced by nitrosamines (5, 25). Indeed, there is some evidence in rats and mice that hepatocytes may be protected from the effects of NMBZA by DNA repair enzymes (14).

In the esophageal mucosa, our results indicate that the ability of the tissue to activate NMBZA plays an important role in determining its toxicity and carcinogenicity. Thus, in the Sprague-Dawley rat, the F344 rat, and the rabbit, there was significant metabolic activity in the esophagus, and these species are susceptible to esophageal carcinogenesis by NMBZA. While it is difficult to compare the potency of NMBZA as an esophageal carcinogen in these animals because of differences in experimental design, for nitrosopiperidine-induced esophageal tumors, the F344 rat is certainly more sensitive than is the Sprague-Dawley rat (19). Our results show that microsomes from the esophageal mucosa of the F344 rat metabolize NMBZA at a greater rate than those from the Sprague-Dawley rat. Furthermore, our observations indicate that, in comparison to the rat, NMBZA shows negligible acute toxicity as well as undetectable levels of debenzylation in the esophagus of the Syrian hamster and BALB/cByJ mouse, again suggesting a correlation between metabolism at the methylene carbon and the biological effects of the nitrosamine in these tissues. In Syrian hamsters, the esophagus is very rarely a target organ for nitrosamines (18), although in BALB/c mice, the esophagus is susceptible to tumor induction by long-term administration of nitrosodimethylamine in drinking water (4). The organ most susceptible to nitrosodimethylamine oncogenesis in BALB/c mice, however, was the forestomach (4) which, like the esophagus, has an undetectable level of NMBZA-debenzylase. In the mouse, therefore, susceptibility of these tissues towards nitrosamines may be highly dependent on the structure of these compounds.

The extremely low level of NMBZA-debenzylase activity we detected in microsomes prepared from human esophageal mucosa is in agreement with the studies of Aurbach and Stoner (2), in which they showed that methylation of DNA by NMBZA in rat esophageal cultures was about 100-fold higher than in cultures of human esophagus. These 2 studies suggest that environmental exposure to NMBZA may play only a minor role in the etiology of human esophageal cancer. However, at present, the extent of DNA modification required for malignant transformation by NMBZA is unknown. Large interindividual variations in rates of metabolism of carcinogens by human tissues have also been demonstrated (9). Furthermore, epidemiological studies have implicated alcohol, smoking, and certain dietary deficiencies as predisposing factors in human esophageal cancer (20, 26, 31, 33). To date, the effects of these various factors on NMBZA metabolism in the human esophagus remain unclear, although recent studies have shown that dietary zinc deficiency can increase the incidence of esophageal tumors in the rat (8) and that pretreatment with alcohol can lead to an enhancement of DNA methylation by NMBZA in the rat esophagus (15).

The low but significant rate of NMBZA activation by rat lung microsomes correlates with our observation of a lack of toxicity in this organ, although weak toxic and tumorigenic effects of NMBZA have been reported by others (16, 30). Whether NMBZA is carcinogenic in the lungs of the Syrian hamster or BALB/cByJ mouse, in which we found comparatively high levels of metabolic activity but no toxicity, remains to be determined. It is of interest that, in gerbils, methylation of DNA by NMBZA was shown to be higher in the lung than in any other tissue (3), but no lung tumors were induced by the nitrosamine (32).

Low levels of NMBZA-debenzylase were detected in kidney microsomes from the Syrian hamster and BALB/cByJ mouse and in forestomach microsomes from the Syrian hamster, but NMBZA displayed no toxicity in these organs. In the Sprague-Dawley rat, the undetectable level of NMBZA metabolism by kidney microsomes correlates with the lack of toxicity of the nitrosamine in this organ.

The present investigations demonstrate that the levels of the microsomal enzyme system responsible for metabolic activation of NMBZA vary considerably between different organs in different species. Although in all species examined, the highest rate of metabolic activation occurs in the liver; this organ is resistant to the toxic or carcinogenic effects of the nitrosamine. Among extrahepatic tissues, only the esophagus, a principal target organ in a number of species, exhibits a positive correlation between metabolic capacity and toxicity or carcinogenicity by NMBZA. Therefore, metabolic activation appears to be a necessary but not sufficient stimulus for the tumorigenic effects of NMBZA.

REFERENCES

Metabolism and Toxicity of NMBZA

Fig. 1. Normal, untreated rat esophagus. H & E, x 250.
Fig. 2. Section of the esophagus from a rat treated i.p. with NMBZA, showing acanthosis of the squamous epithelium with disorganization of basal layers and focal exocytosis. H & E, x 250.
Fig. 3. Esophagus from a rat treated i.p. with NMBZA. Section through the submucosal and muscle layers showing perivascular distribution of plasma cell and lymphocytic infiltration. H & E, x 250.
Fig. 4. Normal, untreated mouse esophagus. H & E, x 250.
Fig. 5. Section of the esophagus from a mouse treated p.o. with NMBZA, showing disorganization of the basal layers. H & E, x 250.
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