Pyrazole Effects on Mutagenicity and Toxicity of Dimethylnitrosamine in Wistar Rats

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ABSTRACT

The correlation between the in vivo toxicity and in vitro mutagenicity of dimethylnitrosamine and the activity of dimethylnitrosamine demethylase I (DMND I) after pyrazole treatment of rats was studied. The biological effects of pyrazole were measured either as toxicity to the rats or as mutations to Salmonella TA 92. A dose-response relationship was observed between DMND I activity and the administered dose of pyrazole. Pyrazole administration increased the toxicity of dimethylnitrosamine when measured as a 50% lethal dose or as a histopathological effect on the liver.

Phenobarbital and methylcholanthrene administration did not have any effect on the activity of DMND I or on the number of histidine-revertant colonies when tested using the liquid suspension method in the presence of dimethylnitrosamine and the reduced nicotinamide adenine dinucleotide phosphate-generating system. When microsomes from the pyrazole-treated animals were used in the mutagenesis assay, there was a linear correlation between DMND I activity and the number of histidine-revertant colonies. It is concluded that pyrazole treatment of animals increases the activity of liver DMND I, the toxicity of dimethylnitrosamine, and the number of mutations.

INTRODUCTION

The metabolism of DMN to the ultimate carcinogenic species is thought to be mediated via a microsomal mixed-function oxidase system (3, 23). The first step in the metabolism is enzymatic hydroxylation of the methyl group. The hydroxymethyl derivative of DMN undergoes nonenzymatic rearrangement to a methylating intermediate with simultaneous liberation of formaldehyde. The microsomal enzyme responsible for this metabolic event is called DMND. Isoenzymes of DMND differ in their affinity for DMN (1, 2, 20). The Km for the high-affinity isoenzyme is between 0.2 and 1.5 mM, and the Km for the low-affinity isoenzyme (DMND I) is thought to be of biological significance. When the relationship between the induction of DMND and the biological effect of active DMN metabolites was studied, correlation between oxidative demethylation of DMN and activation of DMN to a bacterial mutagen was demonstrated at a high substrate concentration of DMN (4, 6, 10, 24, 30). However, the studies with low substrate concentration have produced controversial results. In some cases, demethylation of DMN and its activation to mutagen are regarded as equivalent (13, 14), whereas in other cases no correlation was demonstrated (15). The high-affinity isoenzyme of DMND is not inducible by phenobarbital, methylcholanthrene, or Aroclor 1254 (1). Acetone, isopropyl alcohol, ethanol, and indole (9, 11-13) have been reported to increase the activity of DMND I. Our earlier experiment showed pyrazole to be a potent inducer of DMND I when the activity was measured 6 hr after pyrazole administration (8, 9). Furthermore, pyrazole led to the appearance of a new protein with an approximate molecular weight of 51,000 at the cytochrome P-450 area on sodium dodecyl sulfate-gel electrophoresis (7, 8). Recently, these same observations were independently obtained by Tu et al. (32). Pyrazole derivatives are widely used as antiinflammatory, antipyretic, and analgesic drugs.

In the present work, we measured the extent to which the in vivo toxicity and in vitro mutagenicity of DMN are correlated with the activity of liver DMND I from untreated and pyrazole-treated rats.

MATERIALS AND METHODS

Weanling male Wistar rats were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). They were kept in plastic cages, 5/cage, in a room with a 12-hr light cycle. They received water and food (Wayne Lab Blox) ad libitum. Pyrazole was administered as i.p. injections in 0.9% NaCl solution at the dose levels indicated. Phenobarbital (75 mg/kg body weight) and methylcholanthrene (25 mg/kg body weight) were administered on 3 consecutive days as i.p. injections. The animals were killed by decapitation. The livers were homogenized in a solution containing 10 mM EDTA and 150 mM KCI (pH 7.25) and centrifuged at 10,000 x g for 15 min. The supernatant was centrifuged at 105,000 x g for 1 hr. The microsomal pellet was suspended in 0.1 M phosphate buffer (pH 7.4), and the centrifugation at 105,000 x g was repeated. The incubation mixture for the determination of DMND contained in a total volume of 1 ml: 5 mM MgCl2, 7.5 mM semicarbazide-HCl, 2 mM NADP, 4 mM glucose-6-phosphate, and 2 units glucose-6-phosphate dehydrogenase in 0.1 M potassium phosphate buffer, pH 7.4. The concentration of DMN varied from 0.4 to 4 mM. The amount of the microsomal protein varied from 1 to 2 mg per ml incubation mixture. After a 30-min incubation at 37°C, the formation of formaldehyde was determined by the method of Lowry and others (22).

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Mutagenesis. Salmonella typhimurium TA 92 was a gift from Dr. B. Ames (University of California, Berkeley, Calif.). This Salmonella strain is more sensitive to DMN than are the other strains (25). An overnight culture of the strain in nutrient broth was used for mutation tests. For testing the mutagenicity of DMN, bacteria, microsomal suspension, and a NADPH-generating system were incubated in a shaken 37°C water bath for 30 min as described by Hutton et al. (14). The concentration of cofactors and buffer in 1 ml incubation mixture were the same for the mutagenicity assay as for determination of DMND I. The concentration of DMN and protein varied as indicated in Charts 3 and 4. After

1 To whom requests for reprints should be addressed, National Cancer Institute, Bldg. 37, Room 3B27, Bethesda, Md. 20205.

2 The abbreviations used are: DMN, dimethylnitrosamine; DMND, dimethylnitrosamine demethylase; DMND I, dimethylnitrosamine demethylase I; AHH, arylhydrocarbon hydroxylase; LD50, 50% lethal dose.

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incubation, 2 ml of top agar were added to the incubation mixture. The content of the tube was vortexed and poured onto the minimal agar plate. The number of the histidine-revertant colonies in quadruplicate were counted after 48 hr incubation at 37°.

**Histology.** Specimens of the liver from median and left lateral lobes were fixed in Bouin’s fixative. Paraffin sections were stained with hematoxylin and eosin.

**RESULTS**

Table 1 shows the effect of pyrazole and imidazole on microsomal DMND and AHH. Pyrazole was a better inducer of DMND I than was imidazole, whereas imidazole induced AHH, but pyrazole decreased the activity significantly. Only pyrazole was used in the following experiments as an inducer of DMND I.

The effect of pyrazole treatment (200 mg/kg body weight) on the LD50 level of DMN was studied. DMN was administered to the control and to the pyrazole-treated animals 24 hr after pyrazole administration at 7 different dose levels (6 to 8 animals/group). The number of dead animals was recorded 72 hr after the administration of DMN. When these data were used for probit analysis, the LD50 for control animals was 63.39 mg/kg body weight with 95% confidence limits from 51.45 to 80.47. The corresponding values for the pyrazole-treated animals were 29.26 mg/kg body weight and 95% confidence limits from 10.53 to 40.42.

For histopathological studies, 5 male weanling Wistar rats were given one injection of pyrazole (200 mg/kg) and 24 hr later were given DMN (15 mg/kg body weight). Five control rats were given the same amount of DMN. The animals were killed 3 days later. Centrilobular necrosis was prominent among animals treated with pyrazole, whereas no prominent necrotic areas were present among the controls.

In the next experiment, microsomes from the control, phenobarbital-, methylcholanthrene-, and pyrazole-treated rats were used (Chart 1). Three i.p. injections were given to the animals. Pooled microsomes from each treatment group were used at the enzyme activity was about 3 times higher than the values obtained for the control microsomes. For the control microsomes, the number of revertant colonies at any protein and substrate concentrations varied from 0.4 to 4 mM. Each point represents the average of 4 determinations of the enzyme activity of the pooled microsomal preparations from 5 young male Wistar rats.

**Ames Test.** The values for the histidine-revertant colonies in Chart 2 were obtained using the same microsomal preparations as above. The conditions for the incubation of *S. typhimurium* TA 92 were also the same as those used for the determination of formaldehyde. There were no differences in the number of histidine-revertant colonies when control, methylcholanthrene, or phenobarbital microsomes were used, whereas the microsomes from the pyrazole-treated animals gave a prominent increase in the number of mutations. Chart 3 shows the effect of the different substrate and protein concentrations on the number of revertant colonies from the control and the pyrazole-treated animals. For the control microsomes, the number of revertant colonies at any protein and substrate concentrations

![Pyrazole and Effects of DMN](chart.png)

**Chart 1.** Effects of methylcholanthrene (- - -), phenobarbital (-- --), and pyrazole (-----) on liver DMND activity at different substrate levels of DMN. Protein concentration, 2 mg/ml. Points, average of 4 determinations of the enzyme activity of the pooled microsomal preparations from 5 young male Wistar rats. ---, control microsomes.

**Table 1**

Effect of imidazole and pyrazole on the microsomal enzyme activities of liver.

Animals were given 3 i.p. injections of imidazole or pyrazole (200 mg/kg body weight) on consecutive days. Animals were killed 24 hr after the last injection.

<table>
<thead>
<tr>
<th>DMND (mmol/hr/mg microosomal protein)</th>
<th>AHH (pmol/hr/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 100 ± 5.3 (4)</td>
<td>165 ± 10.4 (8)</td>
</tr>
<tr>
<td>Imidazole 209 ± 17.1 (11)</td>
<td>618 ± 86.6 (7)</td>
</tr>
<tr>
<td>Pyrazole 304 ± 10.3 (7)</td>
<td>99 ± 13.6 (9)</td>
</tr>
</tbody>
</table>

Table 1. Effects of methylcholanthrene (-- --), phenobarbital (-- --), and pyrazole (-----) on liver DMND activity at different substrate levels of DMN. Protein concentration, 2 mg/ml. Points, average of 4 determinations of the enzyme activity of the pooled microsomal preparations from 5 young male Wistar rats. ---, control microsomes.
remained low, usually less than 200 colonies/plate. When pyrazole microsomes were used, the number of the histidine-revertant colonies increased with increased protein and substrate concentrations. However, there was a significant decrease in the number of colonies when 4 μmol DMN per ml were used with 2 mg protein per ml incubation mixture. In addition, there was a lack of precision of the measurement of numbers of revertant colonies. The range of colonies obtained with pyrazole microsomes at 2 mg protein with 0.8 and 4 mM DMN concentrations were, in Chart 2, 539 to 695 and 1826 to 2724. The corresponding values in Chart 3 were 1143 to 1535 and 1471 to 1938. When the values from Table 2 for DMND activity and corresponding values for histidine-revertant colonies were used for correlation analysis, a correlation coefficient of 0.993 (p < 0.01) was obtained.

DISCUSSION

We had shown earlier that pyrazole is a potent inducer of liver high-affinity DMND and that this induction is clearly present 6 hr after one administration of pyrazole (7, 8). The difference between our results and those obtained with pyrazole by Lake et al. (19, 20, 28, 29) may be due to the different timing of determinations after pyrazole administration. In their studies, the time between pyrazole administration and experiment was either 30 min or 1 hr. The inhibitory effect of pyrazole on the metabolism of DMN when the latter is administered shortly after pyrazole treatment. Morphological evidence pointed to a similar effect by pyrazole. The liver necrosis was evident when DMN was administered at 15 mg/kg body weight to pyrazole-treated animals, whereas in the control animals no major necrotic areas could be seen.

Numerous experiments have shown a relationship between the oxidative dealkylation of DMN and the increase in frequencies of histidine-revertant mutations of S. typhimurium when a high concentration of DMN together with Aroclor-, phenobarbital-, or methylcholanthrene-induced microsomal preparations are used in mutagenicity testing (4, 6, 10, 24, 30). Typical microsomal enzyme-inducing agents repress the activity of DMNI (33, 34). Thus, Haag and Sipes (13) observed a significant reduction in the number of revertant colonies when the microsomes from Aroclor-pretreated mice were present in liquid suspension method together with low levels of DMN.

Our data show no effect on microsomal DMNI activity or on the number of histidine-revertant colonies when the animals are treated with phenobarbital or methylcholanthrene. Only pyrazole is a specific inducer for this enzyme, and it increases significantly the number of revertants (Charts 2 and 3). In contrast, it decreases the activity of AHH (Table 1). In general, the number of mutations is dependent on the concentration of DMN and microsomal protein, as shown in Chart 3. However, at the highest protein and DMN concentrations, there is a deviation from linearity. The correlation coefficient for the dependency of histidine-dependent colonies on the microsomal DMND activity is 0.993 (p < 0.01). Thus, our results with pyrazole-induced microsomes show a strong correlation between microsomal DMND activity and the ability to form mutagenic agents from DMN. Similar correlation was obtained by Hutton et al. (14) when microsomes prepared from hamster liver were used. However, for mouse liver microsomes, the correlation is not evident, and higher concentrations of DMN had to be used (15). In a recent publication by Prival and Mitchell (31), inhibitory factors were shown to be present in the cytosolic fraction of the liver, which might explain the differences between the mutagenic activity and the activity of

Inhibition following stimulation of microsomal monooxygenase activities is well known among dioxy compounds such as piperonyl butoxide (16, 17). Furthermore, it has been shown that acetone or isopropyl alcohol increases microsomal N-demethylation 16 and 24 hr after the treatment of the animals, whereas no change was observed 4 hr after the treatment (12).

Our experiments show a clear dose-response curve when the animals received pyrazole at 3 different dose levels. Induction of liver DMND by pyrazole agrees with the decreased LD50 for DMN when DMN administration followed 24 hr after pyrazole treatment. Morphological evidence pointed to a similar effect by pyrazole. The liver necrosis was evident when DMN was administered at 15 mg/kg body weight to pyrazole-treated animals, whereas in the control animals no major necrotic areas could be seen.

Activity of DMND and the number of histidine-revertant colonies at different dose levels of pyrazole to the animals

<table>
<thead>
<tr>
<th>Pyrazole (mg/kg body wt)</th>
<th>No. of animals</th>
<th>DMND (nmol/hr/mg microsomal protein)</th>
<th>Av. no. of His + colonies/plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>146 ± 8.32</td>
<td>399 (287–497)</td>
</tr>
<tr>
<td>100</td>
<td>4</td>
<td>320 ± 9.0</td>
<td>1225 (476–1617)</td>
</tr>
<tr>
<td>150</td>
<td>4</td>
<td>382 ± 12.3</td>
<td>1288 (924–1652)</td>
</tr>
<tr>
<td>200</td>
<td>4</td>
<td>408 ± 22.4</td>
<td>1470 (924–2548)</td>
</tr>
</tbody>
</table>

* Three i.p. injections of pyrazole were given to the animals on consecutive days.

* Mean ± S.E.

* Numbers in parentheses, range.
liver DMND when the 9000 × g supernatant was used (31). Chronic ethanol ingestion and pyrazole have similar effects on liver DMND activity and on microsomal activation of DMN to a mutagen (11).

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REFERENCES

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