THE METABOLISM OF NITROGUANIDINE AND NITROSOGUANIDINE
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The Metabolism of Nitroguanidine and Nitrosoguanidine by Rat Hepatic Subcellular Fractions

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In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

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The metabolism of nitroguanidine and nitrosoguanidine by rat hepatic subcellular fractions was examined. The microsomal fraction and 10,000 g supernatant from untreated and phenobarbital-treated animals and the 9,000 g supernatant from Arochlor-induced rats were used for this study. The in vitro metabolism of substrate was measured by HPLC. The results indicate that neither NG nor NSG are metabolized by the hepatic subcellular fractions.
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The Metabolism of Nitroguanidine and Nitrosoguanidine by Rat Hepatic Subcellular Fractions--Simboli et al

Nitroguanidine, a primary component of US Army triple-base propellants, is now produced in a Government-owned contractor-operated ammunition plant. The US Army Biomedical Research and Development Laboratory (USABRDL), as part of its mission to evaluate the environmental and health hazards of military-unique pollutants generated by US Army munitions manufacturing facilities, conducted a review of the nitroguanidine data base and identified significant gaps in the toxicity data. The Toxicology Branch, LAIR, was tasked by USARRDL to develop a genetic and mammalian toxicity profile for nitroguanidine, related intermediates/by-products of its manufacture, and its environmental degradation products. Nitrosoguanidine is a potential environmental degradation product of nitroguanidine.

The development of a toxicity profile for NG and NsG will aid in understanding the safety or hazards of these compounds. Metabolic fate studies are an integral part of these toxicological tests. To supplement in vivo studies on the fate of nitroguanidine in the rat, this study examines the in vitro metabolism of NG and NsG by hepatic subcellular fractions. Additional impetus to examine the in vitro metabolism of NsG is provided by preliminary reports that NsG was mutagenic in mouse lymphoma assays when activated with Arochlor-induced 9,000 x g supernatant (9K) (1).

The objective of this study is to quantitate the extent of metabolism of NG and NsG by three rat hepatic subcellular fractions: untreated and phenobarbital-treated 10,000 x g supernatant (10K), Arochlor-induced 9,000 x g supernatant, and untreated and phenobarbital-treated microsomal pellet. If the substrates are found to be degraded metabolically, the metabolites will be isolated, identified, and quantified.

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, and Fischer's medium for leukemic cells of mice were
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purchased from the Sigma Chemical Co., St. Louis, MO. Nitroguanidine was supplied by the Sunflower Ammunition Plant, Desoto, KS. The US Army Biomedical Research and Development Laboratory (USABRDL, Fort Detrick, MD) provided the NsG. The Arochlor-induced 9K supernatant was supplied by Microbiological Associates, Bethesda, MD.

Instrumentation

The HPLC system consisted of two LKB 2150 pumps coupled to an LKB 2152 HPLC controller; 20-μl injections were made via a Waters Wisp 710B; the detector was a Perkin Elmer Spectroflow 773; the integrator was a Shimadzu CI-3A. The HPLC column was a Brownlee C-18 Spherisorb column (4.6 mm x 250 mm); a mobile phase of 10% MeOH/H2O was used at a flow rate of 0.7 ml/min; the detector was set at 265 nm (1.000 AUFS). The centrifuges used were a Sorvall RC-5, a Sorvall RC-5 Superspeed Refrigerated centrifuge, and a Beckman L5-75 Ultracentrifuge. A Finemet 100 homogenizer was used to homogenize the livers.

Animals

Male albino Sprague-Dawley rats (354-425 g) were obtained from Bantin-Kingman, Fremont, CA. The inducing agent, sodium phenobarbital (PB), was administered in (80 mg/kg) for four consecutive days prior to sacrifice on day 5. The untreated animals were left untreated during this time period. Animals were fasted for a 24 hour period prior to sacrifice (SOP-OP-STX-88, "Preparation of Hepatic Subcellular Fractions"). The rats were sacrificed by decapitation and the livers were rapidly removed and rinsed in cold 1.15% KCl. The livers were blotted dry, weighed, and placed in a volume of cold 0.1M Tris buffer (pH 7.4) equal to twice the combined weight of the livers. The livers were minced with scissors and homogenized. The homogenate was centrifuged at 10,000 x g (8-10°C) for 20 minutes. The 10,000 x g supernatant was then centrifuged at 100,000 x g for 60 minutes (8-10°C). Following ultracentrifugation, the 100,000 x g supernatant was discarded and the microsomal pellet was resuspended in Tris buffer (3.5 ml/g of liver). The protein content and NADH-demethylase activity were determined (SOP-OP-305-87, "Determination of Total Protein and NADH-Demethylase Activity."). The microsomes and 100,000 x g supernatant were quickly frozen in liquid nitrogen and stored at -70°C refrigeration.

Incubations

Incubations were prepared using five hepatic subcellular fractions: PB induced 9K supernatant,
untreated 10K supernatant, PB-induced microsomes, untreated microsomes, and Arochlor-induced 9K supernatant. The 10K supernatant and microsomal incubations were conducted at a protein concentration of 4 mg/ml. The 2-ml incubates contained 1.0 mM NG or NsG (2 mg/ml DMSO), 5 mM glucose-6-phosphate, 5 mM MgCl₂, 1 unit/ml glucose-6-phosphate dehydrogenase, 0.1M pH 7.4 Tris buffer, and the liver fractions. Incubates also contained 0.5 mM NADP whereas the control incubates did not. Additional experiments in which the enzyme system was heat-inactivated were conducted by immersing the subcellular fraction in a 90°C water bath for 10-15 minutes before adding the other cofactors.

In order to simulate the activating conditions for mouse lymphoma studies, the 9K incubations contained, in a total volume of 2 ml: 8 mg of protein, 1.0 mM NsG, 3.375 mg/ml isocitrate, 0.6 mg/ml NADP, and Fischer's medium for leukemic mouse cells. Control incubations did not contain NADP. Control incubates containing only Fischer's medium and NsG were also prepared.

All incubations were performed in a water bath shaker at 37°C. The incubation period for the 10K and microsomal fractions was 20 minutes. The 9K supernatant fractions were incubated for 2 hours. After the desired incubation period, the postincubates were placed in an ice bath and terminated by the addition of 250 ul of 5% ZnSO₄; 2 umol of the internal standard (IS) methyl nitroquanidine was then added. The incubates were centrifuged for 5 minutes at 4,000 g, and the resulting supernatant was filtered via Millipore Swinnex filtering system (0.45 μ). The filtrate was analyzed via HPLC.

RESULTS

The HPLC traces obtained from the postincubates were clean with no interfering peaks. Nitroguanidine eluted sharply at 4.9 min, NsG eluted at 4.5 min, and the internal standard eluted at 5.8 min. Table 1 shows the HPLC peak area ratios of NG/IS obtained from incubations of nitroguanidine with untreated and PB-induced 10K supernatant and untreated and PB-induced microsomes. No significant difference in the amount of NG remaining in incubations with the NADPH-generating system and in the corresponding control incubations without NADPH was seen. The same results were obtained when the control incubates were heat-inactivated. The standard deviation values give a rough estimate of the precision of the analysis; in the case of NG, the precision is about 5%.
Table 2 shows the HPLC peak area ratios of NsG/IS obtained from incubations of NsG with untreated and PB-treated 10K supernatant and microsomes as well as with commercially obtained Arochlor-induced 9K supernatant. No significant differences are seen in the amount of NsG present in incubations with or without NADP added; again, the same results were obtained when the enzymes of the control incubates were heat-inactivated. Additionally, NsG was not degraded over a longer incubation period (2 hours at 37°C, results not shown). In this case, the precision in the measurement of NsG appears to be in the order of 5-10%.

DISCUSSION

The metabolism of NG and NsG by untreated and phenobarbital-treated rat hepatic 10,000 x g supernatant and microsomal fractions was examined. The disappearance of NG or NsG from these incubations was monitored by HPLC; the results indicate that these compounds do not undergo NADPH-dependent metabolism by rat liver 10,000 x g supernatant or microsomes. This observation is consistent with recent in vivo data that indicate NG to be rapidly absorbed through the gut, to enter the bloodstream, and to be quickly excreted unchanged into the urine; it appears that NG and NsG are not metabolized by the liver but are rapidly passed out of the body (2). No metabolism was seen in incubations conducted with hepatic fractions obtained from PB-induced animals, thus demonstrating that NG and NsG are not subject to hepatic enzyme biotransformation.

Nitrosoguanidine was found to have slight mutagenic capabilities in mouse lymphoma assays (1). The metabolism of NsG was examined under conditions similar to that of the mouse lymphoma assay with Fischer's medium for leukemic cells of mice and 9K supernatant to determine if metabolic activity could assist in explaining NsG mutagenicity. No NADPH-dependent metabolism of NsG was seen in this system. This seems to indicate that the mutagenicity observed in the mouse lymphoma assay is not due to a metabolic or chemical degradation product of NsG. However, it should be noted that, due to the imprecision (CV is less than 5%) of the assay in quantitating NsG, small amounts (up to 10 ug) of NsG may be metabolized and yet go undetected under these conditions. Additionally, possible metabolites of NsG are likely to have low molar extinction coefficients so they could be difficult to detect with an UV detector.
REFERENCES

1. Harbell JW. Personal communication.

TABLE 1

The NADPH-Dependent Metabolism of Nitroguanidine by Untreated and Phenobarbital-Treated (PB) Rat Hepatic Fractions

<table>
<thead>
<tr>
<th></th>
<th>Peak Area Ratio of NG/IS ± SD (n=7)</th>
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<tr>
<td></td>
<td></td>
<td>+NADP</td>
<td>-NADP</td>
</tr>
<tr>
<td>10K Supernatant</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1.10 ± 0.01</td>
<td>1.12 ± 0.01</td>
<td></td>
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<tr>
<td>PB</td>
<td>1.07 ± 0.04</td>
<td>1.03 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>1.02 ± 0.02^a</td>
<td>1.03 ± 0.01^b</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>0.99 ± 0.02</td>
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^a N = 6
^b N = 5
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<tr>
<th></th>
<th>+NADP (n=7)</th>
<th>-NADP (n=7)</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>1.00 ± 0.09</td>
<td>1.00 ± 0.01</td>
</tr>
<tr>
<td>PB</td>
<td>0.86 ± 0.06</td>
<td>0.86 ± 0.07</td>
</tr>
<tr>
<td>Microsomes</td>
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<td></td>
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<tr>
<td>Untreated</td>
<td>0.89 ± 0.02</td>
<td>0.99 ± 0.01</td>
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<tr>
<td>PB</td>
<td>1.33 ± 0.05</td>
<td>1.37 ± 0.02</td>
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<tr>
<td>9K Supernatant</td>
<td>0.96 ± 0.01</td>
<td>0.93 ± 0.01</td>
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N = 5