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ENZYMATIC DENITRIFICATION OF 2-NITROPROPANE IN UNINDUCED MOUSE LIVER MICROSOMES

(Strain differences; microsomal metabolism; 2-nitropropane)

ELIZABETH K. MARKER and ARUN P. KULKARNI*

Toxicology Program, Department of Environmental and Industrial Health, School of Public Health, The University of Michigan, Ann Arbor, MI 48109 (U.S.A.)

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SUMMARY

Hepatic microsomes from 5 strains of untreated mice were tested for the ability to enzymatically cleave the nitro group from 2-nitropropane (2NP). All strains showed significant NADPH-dependent nitrite release at pH 7.6 and pH 8.8. Statistical differences in nitrite-releasing activity between strains were found between BALB and PL/J and ATH strains at pH 7.6. At pH 8.8, BIO.M differed from CD-1 and BALB. These results are in contrast to a report of little or no denitrification activity in uninduced rats and suggest that the 2NP microsomal metabolism may be of greater importance than previously thought.

INTRODUCTION

The nitroalkane 2NP is used extensively in industry as a component of epoxy resins, inks and paints. Several instances of human toxicity have been reported, with effects ranging from headache, nausea and vomiting to hepatic damage and death [1, 2]. A number of animal studies have been performed to evaluate 2NP toxicity [3–8]. The presence of hepatic tumors was observed in rats after 6 months' exposure to 2NP [6]. Following acute intraperitoneal exposure, 2NP was detected in rat liver, while the presence of nitrite was observed in kidney, spleen, heart and lung as well as liver. The production of up to 89% methemoglobin in peripheral blood was also

* To whom correspondence should be addressed.

Abbreviations: G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; 2NP, 2-nitropropane.

noted in treated animals [7]. It was speculated that hepatic biotransformation of 2NP was necessary for release of nitrite, which was then transported to other organs [8].

The results of in vitro experiments reported by Ullrich et al. [9] provide the basis for enzymatic nitrite release from 2NP. The rates of 2NP denitrification observed by these authors were 10 nmol/min/mg protein for hepatic microsomes from phenobarbital-induced rats and 0.5 nmol/min/mg protein for 3-methylcholanthrene-pretreated rats. The activity in uninduced rats was reported to be either very low or undetectable.

The data presented in this communication indicate that denitrification of 2NP may be a species-specific phenomenon. In contrast to the rat [9], hepatic microsomes isolated from untreated mice are capable of performing this reaction, and strain differences exist in the rates of denitrification.

MATERIALS AND METHODS

2NP (94%) was obtained from Aldrich Chemical Co., Milwaukee, WI and was stored in the dark. Glucose-6-phosphate dehydrogenase from yeast (G6PDH), $NADP^+$ and Hepes (*N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid, sodium salt) buffer were purchased from U.S. Biochemical, Cleveland, OH, while D-glucose-6-phosphate (G6P) was acquired from Sigma Chemical Co., St. Louis, MO. All other chemicals used were reagent grade and available commercially.

Male CD-1 mice were obtained from Charles River Breeding Laboratories, Portage, MI. Male PL/J, ATH, BIO.M and BALB mice were acquired from colonies maintained by the Microbiology/Immunology Department, The University of Michigan, Ann Arbor, MI. Mice were maintained on a 12-h light/dark cycle and allowed one week to acclimatize after receipt. Food and water were available ad lib. All mice were 8–14 weeks of age when killed.

Hepatic microsomes were isolated from homogenates (15–20%) in 50 mM Tris, 0.1 mM EDTA and 0.25 M sucrose, pH 7.4, by differential centrifugation. The microsomes were washed by resuspension in homogenization buffer, followed by recentrifugation at $106\,000 \times g$ for 60 min. Only freshly isolated, washed microsomes, resuspended in 0.25 M sucrose, were used for enzyme assay.

After protein determination by the Biuret method [10] microsomes were diluted to a concentration of 2.0 mg/ml with either 0.1 M Hepes buffer, pH 7.6, or 0.1 M Clark and Lubs buffer ($KCl/H_3BO_3/NaOH$), pH 8.8. Test flask A represented the total nitrite release (enzymatic and non-enzymatic) and contained the following components: 1.0 mg microsomal protein; NADPH generating system consisting of 2.5 mM G6P, 0.5 IU G6PDH, and 1.0 mM $NADP^+$; appropriate buffer to a final volume of 1.0 ml. Control flask B contained all the above assay components, except that 0.1 mM mercuric chloride was added to inhibit electron flow from NADPH-cytochrome P-450 reductase to P-450 [11]. The use of mercuric chloride to inhibit

enzymatic activity in the control flask was chosen over the conventional method of NADPH deletion when preliminary experiments suggested that the presence of NADPH and other reducing agents (e.g., 1.0 mM reduced glutathione, 1.0 mM L-cysteine, 1.0 mM mercaptoethanol) suppressed non-enzymatic nitrite release by as much as 61%.

All flasks were pre-incubated for 5 min at 37°C in a water bath with shaking, and the reaction was initiated by the addition of the indicated amount of 2NP. No solvent was used as substrate vehicle. After aerobic incubation at 37°C for 5 min with constant shaking (120 rev./min), the reaction was terminated using the method of Ullrich et al. [9] as modified in our laboratory. A 0.5-ml sample of the reaction mixture was transferred to 0.5 ml ice-cold zinc acetate (0.5 ml in 50% ethanol) followed by the addition of 0.5 ml ice-cold sodium carbonate (0.5 M). The precipitate formed was removed by centrifugation at 4000 rev./min for 5 min. Nitrite in the supernatant was determined by mixing a 0.5-ml sample with 1.0 ml sulfanilamide (2% in 6 N HCl) followed by addition of 0.25 ml *N*-(1-naphthyl)ethylenediamine (0.16% in 6 N HCl). Absorbance at 540 nm was measured after 15 min. Enzymatic nitrite release was quantified by determining the difference in absorbance between test flask A and control flask B and comparing this difference to standard curves prepared with sodium nitrite under identical conditions. Under these conditions, the enzyme activity is linear with respect to time (up to 20 min) and protein concentration (up to 3 mg/ml).

Results are reported as mean specific activity ± SE. Specific activity is expressed

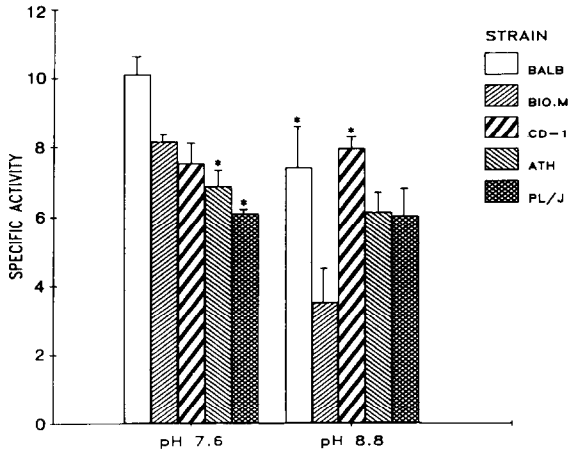

Fig. 1. Hepatic microsomal enzymatic nitrite release from 2NP in 5 strains of untreated mice. Specific activity is expressed as nmol nitrite released/min/mg microsomal protein. Bars represent mean specific activity ± SE for the indicated strain ($N = 3$–5 separate experiments).

* pH 7.6, BALB differs from PL/J and ATH, $P \leqslant 0.05$; pH 8.8, BIO.M differs from CD-1 and BALB, $P \leqslant 0.05$.

as nmol nitrite released/min/mg microsomal protein. Statistical analysis of strain differences at a single pH was by one-way analysis of variance, with Bonferonni comparison of group means [12]. A P value $\leqslant 0.05$ was considered significant.

RESULTS

Enzymatic activity in uninduced mouse liver microsomes was determined under 2 sets of conditions, since preliminary experiments with different buffers using the CD-1 strain had indicated that maximal activity was achieved at pH 7.6 with a 2 NP concentration of 157 mM in 0.1 M Hepes buffer, and at pH 8.8 at a 2NP concentration of 105 in 0.1 M Clark and Lubs buffer. These conditions were used in subsequent experiments.

Enzyme activity was noted in all 5 mouse strains examined. As illustrated in Fig. 1, mean specific activities ranged from 6.1–10.1 nmol of nitrite released/min/mg microsomal protein at pH 7.6, and from 6.0–7.9 nmol of nitrite released/min/mg microsomal protein at pH 8.8, in the different mouse strains.

The highest rate of nitrite release was found at pH 7.6 with microsomes from BALB mice. Significant differences in microsomal nitrite-releasing activity at pH 7.6 were found between BALB mice and 2 other strains: PL/J and ATH ($P \leqslant 0.05$). At pH 8.8, microsomes from CD-1 mice showed the greatest amount of activity (8.0 ± 0.3 nmol nitrite released/min/mg protein). Statistically significant differences at this pH were found only between CD-1 and BIO.M strains, and between BALB and BIO.M strains.

DISCUSSION

In 1978, Ullrich et al. [9] reported a significant P-450-mediated release of nitrite from 2NP by induced rat liver microsomes, but little or no detectable activity in microsomes from control animals. The results presented here, on the contrary, indicate that liver microsomes from untreated mice can catalyze an appreciable amount of nitrite release (Fig. 1) and that nitro-group cleavage from 2NP is not restricted to a particular strain of mouse, but appears to be a species-related phenomenon. This contention is supported by the work of Jonsson et al. [13] which demonstrated nitro-group cleavage from 2-nitro-1-phenylpropane in hepatic microsomes from uninduced rabbits. Such qualitative species differences in metabolic activity have also been reported with other reactions. The rat has been shown to be incapable of chlorphentermine *N*-hydroxylation, and the guinea pig cannot *N*-hydroxylate 2-acetamidofluorene [14]. Quantitative differences in metabolism between species are also seen. For example, *O*-demethylation of *P*-nitroanisole proceeds at a rate 4 times greater in microsomes from mouse than in those from rat [15].

Since Ullrich et al. [9] did not observe nitrite-releasing activity from 2NP in unin-

duced rats, the results presented here suggest that the rat may not be the appropriate animal species in which to study 2NP biotransformation. If human liver is similar to mouse liver in catalyzing this reaction, then hepatic metabolism of 2NP and the accompanying release of nitrite should be regarded as a serious toxicological concern.

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