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SPECIES DIFFERENCE IN THE METABOLIC ACTIVATION OF PHENACETIN BY RAT AND HAMSTER LIVER MICROSOMES

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Summary: Phenacetin is mutagenic in *Salmonella typhimurium* TA 100 when liver 9,000 x g supernatant fractions from PCB-treated hamsters instead of rats are used. A mechanism of the species difference in phenacetin mutagenicity was investigated. By high-performance liquid chromatography analysis, it was found that phenacetin is activated to direct-acting mutagens through N-hydroxylation and deacetylation by hamster liver microsomes. Although no significant species difference was observed in N-hydroxylation, rates of deacetylation were 9 to 150 times higher in hamsters than in rats. The results indicate that the marked species difference in phenacetin mutagenicity is due to the difference in deacetylation activity between rat and hamster liver microsomes.

The rodent species from which liver 9,000 x g supernatant (S9) fractions are obtained is an important factor in the efficient detection of carcinogens as mutagens. Liver S9 fractions prepared from PCB-treated rats have widely been used for the metabolic activation of various promutagens in Salmonella/microsome test (1). It is reported, however, that the mutagenicity of phenacetin, a component of many analgestic drugs, in *S. typhimurium* TA100 is detected only in the presence of liver S9 fractions from hamsters, but not rats (2,3).

Recently, P.J. Wirth et al. and H. Bartsch et al. found that some of putative phenacetin metabolites are mutagenic

ABBREVIATIONS: *S. typhimurium* = *Salmonella typhimurium*, S9 = 9,000 x g supernatant, hplc = high-performance liquid chromatography.

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without activation (4,5,6). Although it is supposed that rats and hamsters have different ability to form these direct-acting mutagens, the exact mechanism of the species difference is still uncertain.

In this study, we investigated the mechanism of the species difference in the metabolic activation of phenacetin by rat and hamster liver microsomes. The results of our quantitative analysis by high-performance liquid chromatography (hplc) indicated that the species difference in phenacetin mutagenicity is due to the difference in deacetylation activity between rat and hamster liver microsomes.

MATERIALS AND METHODS

Materials: N-Hydroxyphenetidine and *p*-nitrosophenetole were synthesized according to the methods of P.J. Wirth et al. and J.T. Hays et al., respectively (5,7). N-Hydroxyphenacetin was synthesized by the method of J.A. Hinson and J.R. Mitchell (8) and identified by comparing its retention time of hplc (TSK LS-410 column, acetonitrile:0.25 mM Na_2HPO_4 (3:7), 1 ml/min, 5°C, Rt 9.9 min) and the mass spectrum (m/z 195) with those of the authentic sample supplied by Dr. K. Shudo of Tokyo University. *p*-Phenetidine was purchased from Wako Pure Chemical Industries Ltd (Osaka) and was purified by distillation (b.p. 134°C/20 mm Hg). Phenacetin and acetaminophen were supplied by Dr. H. Tatsuzawa of National Institute of Hygienic Sciences. The purity of these compounds was checked by hplc under the conditions described above and no definite peaks of contaminants were observable. *S. typhimurium* TA100 was supplied by Dr. B.N. Ames of university of California.

Preparation of liver microsomes: Male Fischer rats (100-120 g) and male Golden Syrian hamsters (80-100 g) were pretreated with a single intraperitoneal injection of PCB (Kanechlor KC-400, 500 mg/Kg) dissolved in olive oil and killed 5 days later. Control animals received olive oil alone. S9 and microsome fractions were prepared as described previously (9).

Assay Methods: The incubation mixture (12 ml) contained 24 mg of hepatic microsomal protein prepared from PCB-treated or untreated rats or hamsters, 600 µmol of potassium phosphate buffer (pH 7.4), 4 µmol of $NADP^+$, 60 µmol of $MgCl_2$, 124 µmol of G-6-P, 8 units of G-6-P dehydrogenase and 16.8 µmol of phenacetin. Sodium fluoride (1.2 mmol) was included in the incubation mixture when phenacetin N-hydroxylation activity was determined. The incubation mixture contained 0.7 µmol of *p*-phenetidine or 5.1 µmol of N-hydroxyphenacetin instead of phenacetin when *p*-phenetidine N-hydroxylation or N-hydroxyphenacetin deacetylation activity was determined. The mixture was incubated for 20 min at 37°C with shaking (100 strokes/min). The incubation time was

shortened to 10 min when N-hydroxyphenacetin deacetylation activity was determined. The reaction was terminated by rapid cooling in an icebath.

Extracts of metabolites and unmetabolized substrate were obtained by extracting the incubation mixture with three 24 ml aliquots of dichloromethane. The organic phases were combined and evaporated under nitrogen, and the residue dissolved in 500 µl of acetonitrile.

The metabolites were analyzed by hplc using a reverse-phase column, Toyo Soda TSK LS-410 (4 mm I.D. x 300 mm) as described previously (10). The column temperature was 5°C and the flow rate was 1 ml/min. The elution was performed with acetonitrile and 0.25 mM Na_2HPO_4 solution gradient system. The solvent program was as follows. From 0 to 7 min, the solvent system was linearly changed from 15% to 24% acetonitrile. From 7 to 30 min, the elution was performed with 24% acetonitrile solution. From 30 to 45 min, the system was linearly changed from 24% to 80% acetonitrile and since 45 min the elution was performed with 80% acetonitrile solution. Each fraction separated by hplc was tested for its mutagenicity without activation by using *S. typhimurium* TA100 as described previously (10). The identification of each metabolite was performed by comparing its retention time of hplc and UV spectrum with those of the authentic sample. The amount of metabolites formed was determined from calibration curves constructed for each authentic sample by using a microcomputer Chromatopak C-R1A (Shimadzu Ltd, Tokyo).

RESULTS AND DISCUSSIONS

To investigate the metabolic activation route, metabolites of phenacetin by PCB-treated hamster liver microsomes were analyzed. As shown in Fig. 1 B, acetaminophen, N-hydroxyphenetidine, N-hydroxyphenacetin, *p*-phenetidine and *p*-nitrosophenetole were identified. When each metabolite including unknown one was tested for its mutagenicity, N-hydroxyphenetidine and *p*-nitrosophenetole were found to be mutagenic in TA100 cells without activation. It was uncertain, however, whether these direct-acting mutagens were formed from *p*-phenetidine or from N-hydroxyphenacetin. Thus, we analyzed the metabolites of *p*-phenetidine and N-hydroxyphenacetin. As shown in Fig. 1 C,D, N-hydroxyphenetidine and *p*-nitrosophenetole were also identified as metabolites of both *p*-phenetidine and N-hydroxyphenacetin.

Based on the results, we suggested that there are two routes for the metabolic activation of phenacetin by hamster liver microsomes (Fig. 2). Phenacetin is initially activated to both

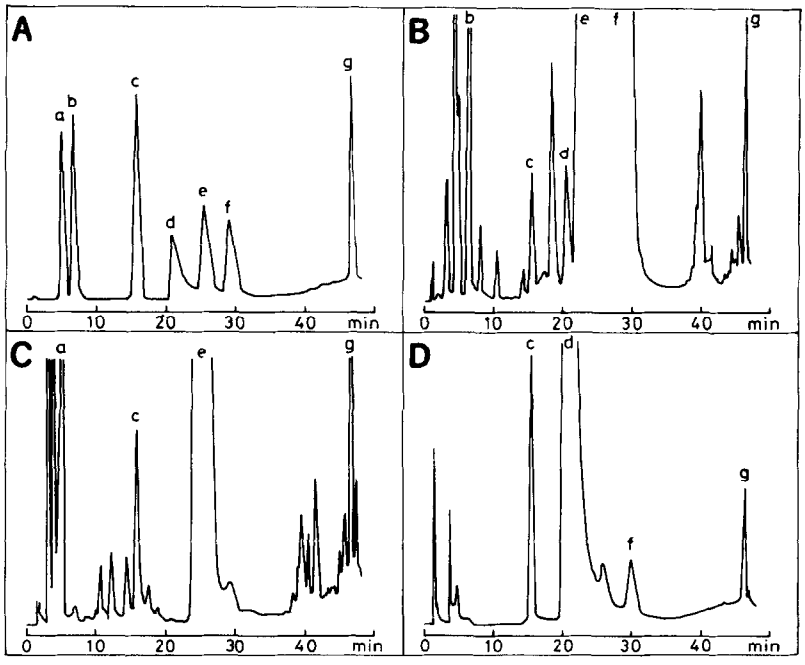

Fig. 1 (A) Chromatogram of authentic samples of phenacetin and its metabolites separated by ODS column (TSK LS-410). The column temperature was 5°C and the flow rate was 1 ml/min. The elution was performed by acetonitrile:0.25 mM Na_2HPO_4 solution ranging from an initial concentration 24:76% to a final concentration 80:20%. The solvent program was described in MATERIALS AND METHODS. This method was applied to the analysis of the metabolites of phenacetin (B), *p*-phenetidine (C) and N-hydroxyphenacetin (D) formed by PCB-treated hamster liver microsomes. When phenacetin deacetylation activity was determined, re-chromatography for 50-fold diluted sample had to be carried out, because the peaks of phenacetin and *p*-phenetidine were unseparated under the standard conditions. The small letters of the alphabet indicate the peaks of *p*-aminophenol (a), acetaminophen (b), N-hydroxyphenetidine (c), N-hydroxyphenacetin (d), *p*-phenetidine (e), phenacetin (f) and *p*-nitrosophenetole (g), respectively.

p-phenetidine and N-hydroxyphenacetin through deacetylation and N-hydroxylation and they are further activated to direct-acting mutagens, i.e., N-hydroxyphenetidine and *p*-nitrosophenetole.

To clarify the cause of species difference in phenacetin mutagenicity, we compared the rates of each activation step mediated by PCB-treated or untreated rat or hamster liver microsomes. As shown in Fig. 3 A,C, N-hydroxyphenacetin or phenacetin deacetylation activity was about 9 times or 150 times higher in hamsters than in rats respectively. PCB treatment had no

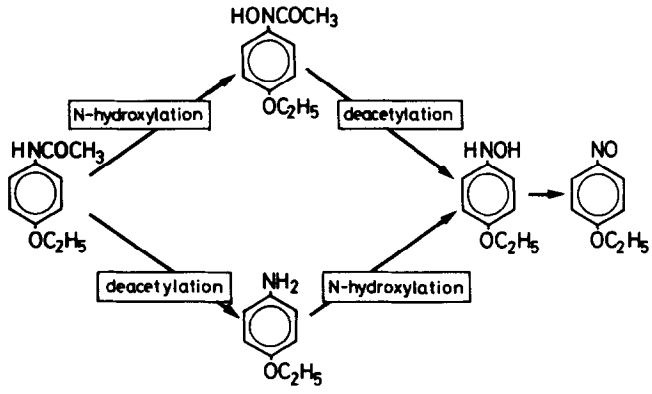

Fig. 2 Proposed mechanism for the metabolic activation of phenacetin by hamster liver microsomes.

significant effect on these activities. Conversely, *p*-phenetidine N-hydroxylation activity increased 3 times in rats and twice in hamsters by PCB treatment and no significant species difference was observed (Fig. 3 B). Phenacetin N-hydroxylation activity also increased 2.5 times in rats and 7 times in hamsters by PCB treatment and the activity in PCB-treated hamsters was 3.8 times higher than that in PCB-treated rats (Fig. 3 D). N-Hydroxyphenetidine and *p*-nitrosophenetole forming activity increased 3 times in hamsters by PCB treatment, but was not detected in rats regardless of PCB treatment (Fig. 3 E).

Both deacetylation and N-hydroxylation are regarded as essential steps for the activation of the known carcinogen, acetylaminofluorene, to bacterial mutagens (11). Our results indicated that phenacetin is also activated to direct-acting mutagens through deacetylation and N-hydroxylation (Fig. 1 B,C, D). Furthermore, the results of quantitative analysis by hplc indicated that both N-hydroxyphenacetin and phenacetin deacetylation activity were significantly higher in hamsters than in rats regardless of PCB treatment and both *p*-phenetidine and phenacetin N-hydroxylation activity increased by PCB treatment regardless of rodent species (Fig. 3 A,B,C,D). It is reasonable

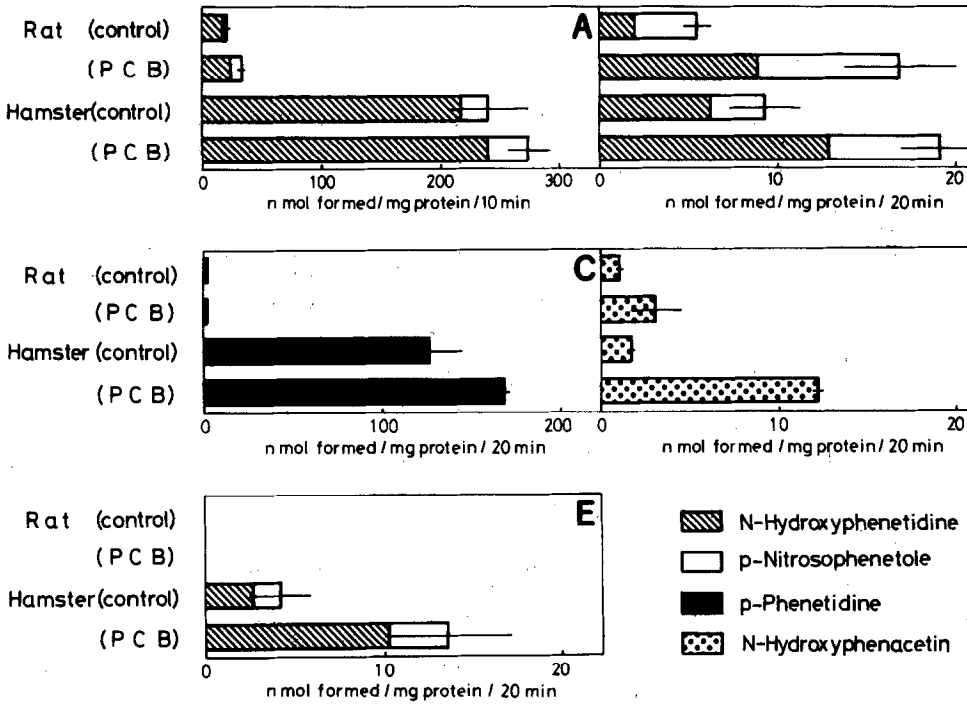

Fig. 3 Comparison of enzyme activity of PCB-treated or untreated rat or hamster liver microsomes. Each graph indicates deacetylation activity of N-hydroxyphenacetin (A) or phenacetin (C) and N-hydroxylation activity of *p*-phenetidine (B) or phenacetin (D). The graph (E) indicates N-hydroxyphenetidine and *p*-nitrosophenetole forming activity. Rates were determined by reference to standard curves prepared with N-hydroxyphenetidine (▨), *p*-nitrosophenetole (□), *p*-phenetidine (■) and N-hydroxyphenacetin (▩). Values are mean ± S.D. of three determinations.

that N-hydroxyphenetidine and *p*-nitrosophenetole forming activity was influenced by both rodent species and PCB treatment (Fig. 3 E), because these direct-acting mutagens were formed through deacetylation and N-hydroxylation (Fig. 2). From the results we concluded that the marked species difference observed in phenacetin mutagenicity is due to the difference in deacetylation activity between rat and hamster liver microsomes.

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