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Effects of Isoniazid and Its Metabolites on Phenytoin Biotransformation in Isolated Rat Hepatocytes

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The inhibitory effects of isoniazid, its metabolites and related compounds on phenytoin biotransformation were studied in an isolated rat hepatocyte system. Oxidation of phenytoin was inhibited strongly by isoniazid, acetylhydrazine, benzoylhydrazine and phenylhydrazine, and weakly (but significantly) by acetylisoniazid and hydrazine. Isonicotinic acid and diacetylhydrazine showed no effect. These observations may indicate that active hydrazine compounds are ones which can give a relatively significant rise to reactive intermediates such as radicals, diazene compounds or their relatives during the oxidation metabolism. On the other hand, glucuronidation of the oxidized metabolite, 5-phenyl-5-(*p*-hydroxyphenyl)hydantoin, was not affected by isoniazid.

Keywords—phenytoin monooxygenation; inhibition; isoniazid; acetylhydrazine; hydrazine; isolated rat hepatocyte; microsomal cytochrome P-450; difference spectrum; spin trapping; glucuronidation

Isoniazid (INH) can inhibit the microsomal mixed function oxidase activity, leading to a reduction of the clearance and an elevation of the plasma level of a co-administered drug.¹⁾ In such cases, drug intoxication may develop in patients. The interactions between INH and phenytoin (PHT) have been well studied. The first clinical cases were reported by Murray in 1962; he found that PHT intoxication symptoms developed in approximately 10% of 630 epileptic patients when they were given INH for the prophylaxis of tuberculosis.²⁾ Later, Kutt *et al.*³⁾ and Buttar *et al.*⁴⁾ found that the formation of the pharmacologically inactive oxidation metabolite, 5-phenyl-5-(*p*-hydroxyphenyl)hydantoin (5-HPPH), was inhibited dose-dependently by co-administration of INH. However, the exact mode of inhibition has still not been fully elucidated, although a spectral investigation of the inhibition on cytochrome P-450 by INH was conducted using rat hepatic microsomes by Muakkassah *et al.*⁵⁾ We examined the effect of INH and some of its metabolic intermediates on the biotransformation of PHT⁶⁾ by using isolated rat hepatocytes, which retain the fundamental metabolic functions of the whole liver.

Experimental

Materials—INH, isonicotinic acid (INA), acetyl hydrazine (AcHz), hydrazine (Hz) sulfate, benzoylhydrazine (BzHz), phenylhydrazine (PhHz) hydrochloride, PHT, 5-HPPH and α -phenyl-*tert*-butylnitrone (PBN) were obtained commercially. Acetylisoniazid (AcINH) and diacetylhydrazine (DAcHz) were prepared by the acetylation of INH and AcHz with acetic anhydride. Collagenase (*Clostridium histolyticum*) was purchased from Boehringer Mannheim GmbH. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Oriental Yeast Co., Ltd. All chemicals were of reagent grade.

Preparation and Incubation of Isolated Rat Hepatocytes—Isolated hepatocytes were prepared from male Wistar rats (180–220 g) according to the collagenase perfusion method as described by Moldéus *et al.*⁷⁾ The viability judged by the lactic dehydrogenase (LDH) latency test⁸⁾ was 98–99% for all preparations. Incubation of the hepatocytes was performed at 37 °C in rotating round-bottomed flasks under a 95%O₂–5%CO₂ atmosphere at a cell concentration of 3×10^6 cells/ml in a Krebs–Henseleit buffer, pH 7.4, supplemented with 13 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) and 10 mM glucose. To obtain the time-courses of PHT elimination and 5-HPPH formation in the presence of INH, each substrate was used at 80 μ M. For the examination of the effect on glucuronidation of 37 μ M 5-HPPH, 36.5 and 73.0 μ M INH were employed.

Sample Preparations and Assay—After incubation for an appropriate period, 0.1 ml aliquots of the mixture were added to 0.3 ml of acetonitrile solution containing 2 μ g of phenacetin as an internal standard. 5-HPPH glucuronide was determined as 5-HPPH after heating of the incubation mixture with 3 N HCl at 100 °C for 1 h. PHT and 5-HPPH were assayed by using a Shimadzu LC3-A high-performance liquid chromatograph equipped with a Chemcosorb 3-ODS-H column (75 $\times$ 4.6 mm; Chemco Scientific Co.) and a ultraviolet detector (SPD-2A). The mobile phase was a 33:67 (v/v) mixture of acetonitrile–0.05 M phosphate buffer (pH 6.5). The flow rate was 1.0 ml/min and the eluate was monitored at 210 nm.

Microsomal Cytochrome P-450 Determination—Microsomes were isolated from livers of male Wistar rats (200–250 g) according to Ernster *et al.*⁹⁾ The rats were either untreated or received phenobarbital (three daily i.p. injections of 80 mg/kg) prior to sacrifice. Protein content was determined according to the method of Lowry *et al.*¹⁰⁾ and cytochrome P-450 content in rat liver microsomes according to Matsubara *et al.*¹¹⁾ The effect of INH, AcHz or Hz on cytochrome P-450 was estimated under the conditions described in the legend to Table II.

Spectral Investigation of AcHz with Rat Liver Microsomal Cytochrome P-450—The difference spectrum of AcHz with cytochrome P-450 was recorded at room temperature (20 ± 2 °C) on a Shimadzu MPS-2000 spectrometer. The experiment was performed in the reaction mixture described in the legend to Fig. 2.

Spin Trapping of Acetyl Radical by PBN during Microsomal Metabolism of AcHz—The reaction mixture, containing 5.6 ml of microsomal suspension (4.4 mg/ml), 2.0 ml of PBN solution (25 mM), 0.2 ml of AcHz solution (10 mM) and 0.2 ml of NADPH solution (1 mM), was incubated at 37 °C for 20 min, and extracted with benzene. Electron spin resonance (ESR) and mass spectral measurements of the extract were performed by the same method as used for the assignment of hydrazine radical.¹⁴⁾

Results and Discussion

The effect of INH on PHT elimination through metabolic oxidation was investigated by using an isolated rat hepatocyte system. The effects of the following compounds were also examined: AcINH, INA, Hz, AcHz and DAcHz (metabolites of INH), and BzHz and PhHz (structurally similar to INH). As shown in Fig. 1-A, INH, AcHz, BzHz and PhHz inhibited PHT elimination strongly, whereas AcINH and Hz showed a relatively weak (but significant) inhibition. On the other hand INA and DAcHz did not have any effect on the elimination rate of PHT (Fig. 1-B). The results are consistent with the simultaneous formation of 5-HPPH, during the first 20 min period of incubation (Table I), in that the formation of 5-HPPH was retarded markedly by INH and AcHz, almost completely by BzHz and PhHz, and slightly but significantly by AcINH and Hz. The rate of 5-HPPH formation was not affected by INA or DAcHz, as expected.

The main course of PHT metabolism is known to be oxidation followed by glucuronidation.¹²⁾ Muakkassah *et al.* investigated the mechanism of the inhibitory action of INH on microsomal drug metabolism.⁵⁾ They found that the addition of INH to rat liver microsomes

Fig. 1. Effects of INH-Related Compounds on PHT Elimination in Isolated Rat Hepatocytes (80μ M/ 3×10^6 cells/ml)

(A): ○, control; ■, INH; ●, PhHz; ▲, BzHz; ●, AcHz; △, Hz; □, AcINH. (B): ○, control; ▲, DAcHz; ■, INA.

TABLE I. Effects of INH and Related Compounds on 5-HPPH Formation

INH and related compounds	5-HPPH formation ^{a)}	Inhibition (%)	Statistical significance
Control	31.7 ± 0.4	0.0	—
INH	4.7 ± 0.3	85.1	$p < 0.001$
PhHz	0.8 ± 0.1	97.5	$p < 0.001$
BzHz	2.2 ± 0.4	93.2	$p < 0.001$
AcHz	6.7 ± 0.7	79.0	$p < 0.001$
Hz	26.6 ± 1.1	16.3	$p < 0.02$
AcINH	28.6 ± 1.7	10.0	$p < 0.05$
INA	31.3 ± 1.1	1.2	NS
DAcHz	30.1 ± 1.6	5.2	NS

Each initial substrate concentration was 80 μ M. Each value represents the mean $\pm$ S.D. of three experiments. Results were compared by means of Student's *t*-test. ^{a)} μ M/3 $\times$ 10⁶ cells/ml/20 min. NS: Not significant.

Fig. 2. Difference Spectra Produced by Interaction of AcHz with Cytochrome P-450

Rat liver microsomes were suspended in potassium phosphate buffer solution (pH 7.4), and 5.6 ml of the suspension (4.4 mg/ml) was divided into two cuvettes. After recording a flat base-line, 0.1 ml of AcHz solution was added at a concentration of 0.1 mM to the sample cuvette and an equal volume of buffer was added to the reference cuvette. The tracings A, B and C represent the difference spectra recorded 2, 4 and 6 min after the addition of NADPH solution (1.0 mM) to both cuvettes.

(A), 2 min; (B), 4 min; (C), 6 min.

produced an immediate decrease in the binding of carbon monoxide to reduced cytochrome P-450, which could contribute to the inhibition of the mixed function oxidase system by INH. Nicotinic acid hydrazide and BzHz had the same effect, whereas AcHz was inactive. From these findings, they suggested that the hydrazine moiety ($-\text{NHNH}_2$) bearing an aroyl group is a dominant functional group in the inhibition of cytochrome P-450. Our results also indicate that the free hydrazine terminal is an essential functional group for the inhibition of PHT monooxygenation. However, the presence of an aroyl group is not essential. The oxidation of PHT was inhibited almost completely by PhHz, strongly by AcHz and weakly (but significantly) by Hz itself, and these are not aroyl compounds (Fig. 1-A, Table I). It was reported that the oxidation of PhHz with microsomal cytochrome P-450 gave phenyl diazene through the formation of a radical intermediate.¹³⁾ Our group has also found that Hz is oxidized via the successive formation of hydrazine radical ($\text{H}_2\text{N}\dot{\text{N}}\text{H}$) and diimide ($\text{NH}=\text{NH}$) during incubation in rat liver microsomal suspensions, as well as in isolated rat hepatocytes.¹⁴⁾ In addition, it was found that Hz produces an NADPH-dependent difference spectrum characterized by a maximum at 448 nm, which can be inhibited by metyrapone. This suggested the formation of a complex of diimide and rat liver cytochrome P-450.

As for the interaction of AcHz with rat liver cytochrome P-450, Muakkassah *et al.*

TABLE II. Effects of INH, AcHz and Hz on Cytochrome P-450 Contents in Rat Liver Microsomes

Substrate concentration (mM)	Cytochrome P-450 content (%)		
	INH	AcHz	Hz
0	100	100	100
0.5	87.9 ± 1.7 ^{a)}	92.3 ± 1.2 ^{b)}	86.4 ± 0.8 ^{a)}
1.0	85.6 ± 1.7 ^{a)}	89.0 ± 2.9 ^{b)}	85.6 ± 1.6 ^{a)}

The reaction mixture, containing 2.8 ml of microsomal suspension (3.5 mg/ml), 0.2 ml of the substrate solution and 1.0 ml of NADPH solution (1.0 mM), was incubated at 37 °C for 10 min, and the cytochrome P-450 contents were measured according to Matsubara *et al.*¹¹⁾ The control value of 1.67 ± 0.03 nmol/mg protein is expressed as 100%. Each value represents the mean ± S.E. of 3–5 experiments. a) $p < 0.01$, b) $p < 0.05$.

Fig. 3. Effect of INH on 5-HPPH Glucuronidation in Isolated Rat Hepatocytes

Initial concentration of 5-HPPH: 37 μM. ○, control; △, 36.5 μM INH; □, 73.0 μM INH.

reported that AcHz was inactive towards cytochrome P-450.⁵⁾ However, AcHz gave a fairly stable NADPH-dependent difference spectrum characterized by maxima at 451 nm (sharp) and 495 nm (broad), as shown in Fig. 2. Pretreatment of the microsomes with metyrapone (0.02 M) decreased $\Delta A_{451-540}$ to 36.9% and $\Delta A_{495-540}$ to 89.0% of each control value. Pretreatment with PBN (5.0 mM) instead of metyrapone lowered $\Delta A_{451-540}$ to 86.3% and $\Delta A_{495-540}$ to 58.9% of the control values, respectively. It may be concluded that the peak at 451 nm arises from the cytochrome P-450 complex with a diazene-type metabolite (RN=NH), and the absorption at 495 nm is due to acetyl radical (CH₃CO). Acetyl radical formation is also supported by the fact that the mass spectrum of the benzene extract of the microsomal oxidation mixture of AcHz in the presence of a spin trapping agent, PBN, clearly showed the formation of the corresponding PBN-adduct (m/z 220). Furthermore, as shown in Table II, cytochrome P-450 contents in the microsomal suspensions were significantly decreased by the addition of AcHz, Hz or INH. These results argue against the conclusion of Muakkassah *et al.* concerning the inactivity of AcHz towards cytochrome P-450.⁵⁾

These observations may imply that active hydrazine compounds are the ones which can give a relatively significant rise to reactive intermediates such as radicals, diazene derivatives, or their relatives, which probably participate in the inhibitory effect on the mixed function oxidase systems responsible for PHT oxidation. AcINH can be hydrolyzed metabolically to

afford active AcHz and inert INA,⁶⁾ which could explain the relatively weak inhibitory effect of AcINH.

Buttar investigated the biliary excretion of PHT and its metabolites in rats, and suggested that administration of INH inhibited the glucuronidation of 5-HPPH as well as the *p*-hydroxylation of PHT.^{4a)} Isolated hepatocytes, which retain a variety of fundamental metabolic functions including glucuronidation, were used to elucidate whether PHT intoxication is related to the inhibition of 5-HPPH glucuronidation by INH, which may result in the induction of a negative feedback on the formation of 5-HPPH.¹⁵⁾ INH, however, had no effect on the conjugation (Fig. 3).

The results mentioned above probably indicate that the delay in PHT elimination was predominantly due to the inhibition of the monooxygenation of PHT by INH and its active metabolites possessing a free hydrazine terminal or their biotransformed active ones. Further investigations are in progress.

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References

- 1) V. C. Valsalan and G. L. Cooper, *Brit. Med. J.*, **285**, 261 (1982); G. Sutton and H. J. Kupferberg, *Neurology*, **25**, 1179 (1975); A. R. Rosenthal, T. H. Self, O. E. Baker and R. A. Linden, *J. Am. Med. Assoc.*, **238**, 2177 (1977); H. R. Ochs, D. J. Greenblatt, G. M. Roberts and H. J. Dengler, *Clin. Pharmacol. Ther.*, **29**, 671 (1981); R. R. Millar, J. Porter and D. J. Greenblatt, *Chest*, **75**, 356 (1979); D. R. Witmer and W. A. Ritschel, *Drug Intell. Clin. Pharm.*, **18**, 483 (1984).
- 2) F. J. Murray, *Am. Rev. Resp. Dis.*, **86**, 729 (1962).
- 3) H. Kutt, K. Verebely and F. McCowell, *Neurology*, **18**, 706 (1968); H. Kutt, R. Brennan, H. Dehejia and K. Verebely, *Am. Rev. Resp. Dis.*, **101**, 377 (1970).
- 4) a) H. S. Buttar, *Res. Commun. Chem. Pathol. Pharmacol.*, **18**, 35 (1977); b) H. S. Buttar, L. T. Wong and J. H. Moffatt, *Arch. Int. Pharmacodyn. Ther.*, **235**, 9 (1978).
- 5) S. F. Muakkassah, W. R. Bidlack and C. T. Yang, *Biochem. Pharmacol.*, **30**, 1651 (1981).
- 6) W. W. Weber and D. W. Hein, *Clin. Pharmacokin.*, **4**, 401 (1979) and references cited therein.
- 7) P. Moldéus, J. Horberg and S. Orrenius, *Methods in Enzymol.*, **52**, 60 (1978).
- 8) J. Högberg and A. Kristoferson, *Eur. J. Biochem.*, **71**, 77 (1977); D. Köster-Albrecht, H. Kappus and H. Remmer, *Toxicol. Appl. Pharmacol.*, **46**, 499 (1978).
- 9) L. Ernster, P. Siebivity and G. Palade, *J. Cell Biol.*, **15**, 541 (1962).
- 10) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 11) T. Matsubara, M. Kōike, A. Tsuchi, Y. Tochino and K. Sugeno, *Anal. Biochem.*, **75**, 596 (1976).
- 12) N. Gerber, W. L. Weller, R. Lynn, R. E. Rangno, B. J. Sweetman and M. T. Bush, *J. Pharmacol. Exp. Ther.*, **178**, 567 (1971); T. Inaba and T. Umeda, *Drug. Metab. Dispos.*, **3**, 69 (1975).
- 13) H. G. Jonen, J. Werringloer, R. A. Prough and R. W. Estabrook, *J. Biol. Chem.*, **257**, 4404 (1982).
- 14) A. Noda, H. Noda, K. Ohno, T. Sendo, A. Misaka, Y. Kanazawa, R. Isobe and M. Hirata, *Biochem. Biophys. Res. Commun.*, **133**, 1086 (1986).
- 15) P. Borondy, T. Chang and A. J. Glazko, *Pharmacologist*, **31**, 582 (1972).