Participation of P450-Dependent Oxidation of Isoniazid in Isonicotinic Acid Formation in Rat Liver

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By determining the formation amount of isonicotinic acid (INA) from isonicotinic acid hydrazide (isoniazid: INH) in isolated rat hepatocytes, we were able to identify the involvement of the oxidative cleavage of the acid hydrazide. INA formation from INH increased significantly using the isolated hepatocytes prepared from rats pretreated with phenobarbital (PB), 3-methylcholanthrene (3MC), dexamethazone (DEX) and rifampicin (RIF), respectively, in comparison to the control group. On the other hand, a remarkable decrease in INA formation from INH was observed by the addition of such P450 inhibitor as metyrapone or cinetidine as well as an amidase inhibitor bis[p-nitrophenyl]phosphate (BNPP) to the isolated hepatocytes prepared from PB-pretreated rats. By further experiments using rat hepatic microsomes, the oxidative pathway of INA formation in INH metabolism was determined to be P450-dependent, since NADPH and oxygen were both essential for the oxidative pathway of INH to INA and the amount of INA formation was also significantly increased by P450 inducers. Regarding acetylsalicylic acid (AcINH) and isonicotinic acid amide (INAAs), however, INA formation by P450 was little observed in the microsomal experiments.

Key words isonicotin (INH); isonicotinic acid (INA); P450; oxidation; rat; liver

Isonicotinic acid hydrazide (isoniazid: INH), an effective tuberculostat has been employed as a first-line drug all over the world since 1952. 1,2 Therefore, countless papers have appeared regarding INH metabolism. 3,4 Nonetheless, it is difficult to find papers reporting the use of isolated rat hepatocytes in INH metabolism, especially in the estimation of isonicotinic acid (INA) formed from INH as a metabolite. During our investigations of INH-induced hepatotoxicity and drug metabolism using isolated rat hepatocytes, we obtained novel and important information on INH metabolism: the formation amount of INA, which is a stable metabolite of INH including an inert pyridine ring, was significantly increased using the hepatocytes pretreated with such classical P450 inducers as phenobarbital (PB) or 3-methylcholanthrene (3MC). 5 Therefore, in order to confirm the presence of the oxidative pathway in INH metabolism, the present study was undertaken using rat hepatic microsomes as well as isolated rat hepatocytes.

MATERIALS AND METHODS

Chemicals INH and INA were both purchased from Nakalai Tesque Inc. (Kyoto, Japan) and were purified by recrystallization from ethanol before the experiment. Sodium PB was obtained from Tokyo-Kasei Kogyo (Tokyo, Japan). Rifampicin (RIF), dexamethazone (DEX) and 3MC were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), 2-Methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), and bis[p-nitrophenyl] phosphate (BNPP) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.). NADP, NADPH, glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-P DH(Y)) employed in the NADPH generating system were obtained from Oriental Yeast Co., LTD. (Tokyo). Collagenase and isonicotinic acid amide (INAAs) were obtained from Wako Pure Chemical Ind., LTD. (Osaka, Japan). AcINH was prepared in our laboratory. Cimetidine was supplied by Fujisawa Pharm. Co., LTD. (Osaka). All other chemicals were of reagent grade.

Animal Pretreatment Male Wistar rats (Japan SLC Inc., Shizuoka, Japan) weighing from 230—260 g (8 weeks of age) were used for this study. Pretreatment was performed by a respective P450 inducer as follows: 80 mg/kg of PB in saline for 3 d (i.p.), 20 mg/kg of 3MC in corn oil for 3 d (i.p.), 30 mg/kg of RIF in corn oil for 6 d (i.p.), or 250 mg/kg of DEX in corn oil for 3 d (i.p.).

Preparation of Isolated Rat Hepatocytes and Rat Hepatic Microsomes Isolated rat hepatocytes were obtained according to the collagenase perfusion method reported by Moldéus et al. 12 Cells with a viability of more than 95% as determined by LDH (lactic dehydrogenase) latency test and by trypan blue exclusion test were employed. Rat hepatic microsomal fractions were prepared according to the method of Omura and Sato. 13 The protein concentrations were determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using bovine serum albumin (BSA) as a standard. The contents of P450 were determined according to the method reported by Matsubara et al. 14

Incubation and Sample Preparation for HPLC The experimental conditions were determined by the preliminary test in all cases.

1. Incubation in Isolated Rat Hepatocytes Freshly prepared hepatocytes (8×10 6 cells/ml) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 1% BSA, 10 mm glucose, 8% amino acid mixture and 13 mm HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid). At 3 min after the preincubation, the suspension was incubated at 37°C with a substrate (0.5 mM) in rotating round-bottomed flasks under a stream of 95% O2—5% CO2 gas.

2. Incubation in Rat Hepatic Microsomes One milliliter of the suspension of hepatic microsomes (2.0 mg protein/ml) in the NADPH generating system containing 10 mm G-6-P, 2 units/ml G-6-P DH, 4 mm NADP, 10 mm MgCl2, and 100 mm potassium phosphate buffer (K-P buffer, pH 7.4) solution was preincubated for 3 min at 37°C. The incubation
was performed for 20 min after the addition of a substrate (0.3 mM).

3 Sample Preparation for HPLC Both in isolated rat hepatocytes and in rat hepatic microsomes, the reaction was stopped by adding 3 ml of a mixed solution of ethyl acetate and n-butanol (2:1, v/v) to the sample tube, and the sample solution was added with 0.3 ml of tetra-n-butylammonium hydroxide solution (0.5 mol/l) and 0.1 ml of 6-methylnicotinic acid (0.3 mM; internal standard). After the mixture was shaken for 20 min followed by centrifugation for 15 min at 850 × g, 2.5 ml of the upper organic layer was transferred to a tube which contained 1.0 ml of 0.2% hydrobromic acid. After shaking and centrifuging again in the above manner, the upper organic layer was removed by suction with a vacuum aspirator. The lower aqueous solution remaining was frozen at −20°C until analysis. Sample preparations for HPLC were performed by the method reported.[1]

Determination of INA by HPLC The HPLC apparatus consisted of a UV spectrophotometric detector (SPD-10A), a liquid chromatograph (LC-9A) and a chromatopac (CR-6A) (Shimadzu, Kyoto), and a column (TSKgel ODS-80TM, 4.6 mm i.d. × 250 mm, Tosoh, Tokyo). The mobile phase was a mixed solution of 100 mM potassium dihydrogenphosphate solution and methanol (98:2, v/v). The flow rate was 0.8 ml/min. UV detection was performed at 265 nm. In isolated rat hepatocytes, a small amount of INA conjugates such as pyruvate and/or α-ketoglutarate formed under the experimental conditions. However, since these INA conjugates produced in the reaction mixture easily to decompose to INA during the sample preparation, they are usually determined as INA. The amount of AcINH formed was also determined. However, since INA formation from AcINH is P450-independent, there is no need to discuss it here. Thus the formation amounts of AcINH are not indicated.

Effects of P450 Inducers on INA Formation in the Isolated Rat Hepatocyte System INH (0.5 mM) was incubated at 37°C in isolated rat hepatocytes (8 × 10⁶ cells/ml) prepared from the liver pretreated with such P450 inducers as PB, 3MC, Rif and DEX, respectively. Isolated rat hepatocytes pretreated with the corresponding vehicle were used as the control sample. The degree of induction was assessed by determining the amount ofINA formation in comparison to the control group.

Effects of P450 Inhibitors and an Amidase Inhibitor on INA Formation in the Isolated Rat Hepatocyte System INH (0.5 mM) was incubated at 37°C together with such P450 inhibitors as metyrapone (0.02 mM) or cimetidine (0.5 mM), and an amidase inhibitor, BNPP (0.1 mM)[2] in the isolated hepatocytes (8 × 10⁶ cells/ml) prepared from PB-treated rats. The control data were obtained from the sample in the absence of an enzyme inhibitor. The effect of such enzyme inhibitors was then assessed based on the amount of INA in comparison to the control group.

Spectral Investigation of INH or INAA with Rat Liver Microsomal P450 The difference spectrum of INH or INAA with P450 was recorded at 20 ± 2°C on an MPS-2000 spectrometer (Shimadzu, Tokyo). The experiment was performed in the reaction mixture described in the legend of Fig. 4.

Effects of NADPH and Oxygen on INA Formation in Rat Hepatic Microsomes INH (0.3 mM) was aerobically incubated at 37°C for 20 min using rat hepatic microsomes (2.0 mg protein/ml) in the NADPH generating system which consisted of 100 mM of potassium-phosphate buffer (K-P buffer) solution (pH 7.4) containing NADP (4 mM), G-6-P (10 mM), MgCl₂ (10 mM) and G-6-P DH (2 units/ml). After preincubation for 3 min, the control data were obtained in the absence of NADPH or oxygen. The effect of NADPH or oxygen on the oxidative pathway of INA was then evaluated by determining the amount of INA at 20 min after incubation in comparison to the control group.

Effects of PB and 3MC on INA Formation in Rat Hepatic Microsomes INH, AcINH and INAA were all employed as a substrate. The substrate (0.3 mM) was incubated at 37°C for 20 min using rat hepatic microsomes (2.0 mg protein/ml) in the NADPH generating system mentioned above. The control data were obtained from the system using untreated rat microsomes. The effects of PB and 3MC on INA formation from INH, AcINH and INAA in the rat microsomes were then assessed by the amounts of INA formation.

Statistical Analysis A statistical analysis was performed by means of analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The main metabolic pathway of INA formation from INH and INAA is understood to be hydrolysis catalyzed by an amidase as shown in Chart 1.[15,10] During the investigation of INH-induced hepatotoxicity using the hepatocytes prepared from PB- and 3MC-treated rats by HPLC, we observed the participation of P450 in INA formation from INH. As shown in Fig. 1, a remarkable difference between INH and INAA was noticed in INA formation. The amount of INA formed from INH was increased using the same hepatocytes freshly prepared from both PB- and 3MC-treated rats. However, INAA was more difficult in accepting hydrolysis than INH, and remarkable increases by the enzyme inducers similar to those appearing in INH were not observed.

To elucidate the difference in the pattern of enzyme induction between INH and INAA, the effects of P450 inducers on INA formation from INH were also examined in more detail. As shown in Fig. 2A, the hepatocytes prepared from PB- and 3MC-treated rats demonstrated reproducible results with a
Fig. 1. INA Formation in Isolated Hepatocytes Prepared from Untreated, PB-Treated or 3MC-Treated Rats
(A) INH ○, untreated; △, PB-treated; □, 3MC-treated. (B) INAA ●, untreated; ▲, PB-treated; ■, 3MC-treated. INH (or INAA) (0.5 mM) was incubated at 37°C in isolated rat hepatocytes (8×10⁶ cells/ml). The isolated hepatocytes were prepared from untreated, PB-treated (80 mg/kg/d, i.p., 3 d) and 3MC-treated (40 mg/kg/d, i.p., 3 d) rats, respectively. Each point represents the mean ± S.D. (n = 3) (* p < 0.01, ** p < 0.001).

Fig. 2. Effect of P450 Inducers on INA Formation from INH in Isolated Rat Hepatocytes
A. ○, untreated; ▲, PB-treated; ■, 3MC-treated. B. ○, vehicle-treated; △, RIF-treated; □, DEX-treated. INH (0.5 mM) was incubated at 37°C in isolated hepatocytes (8×10⁶ cells/ml). A: The isolated hepatocytes were prepared from untreated, PB-treated and 3MC-treated rats, respectively. B: The isolated hepatocytes were prepared from corn oil (vehicle)-treated (1 ml/kg/d, i.p., 6 d) (○), rifampicin (RIF)-treated (30 mg/kg/d in corn oil, i.p., 6 d) (△) and dexamethasone (DEX)-treated (250 mg/kg/d in corn oil, i.p., 3 d) (□) rats, respectively. Each point represents the mean ± S.D. (n = 3) (* p < 0.01, ** p < 0.001).

remarkable increase in INA formation: these findings show a significant increase to 1.7 and 4.1 times above that in the untreated group, while the DEX- and RIF-treated groups indicated a similar tendency regarding the increase of INA formation to 3.2 times that for DEX and to 1.7 times that for RIF in comparison to the respective untreated groups (Fig. 2B).

Further investigation in the hepatocytes was performed using such P450 inhibitors as metyrapone and cimetidine, and an amidase inhibitor, BNPP. As shown in Fig. 3, these enzyme inhibitors significantly inhibited the INA amount to 28.6% of the control value at 40 min after the addition of metyrapone, to 21.4% for cimetidine and to 80.9% for BNPP, respectively. Especially, the inhibition by BNPP of INA formation from INH, was thus strong, while the hydrolysis of INAA was completely inhibited. Metyrapone and cimetidine inhibited the INA formation weakly but clearly. These findings suggested that a small but very significant extent of P450-dependent oxidation participates in INA formation, although a major alternate route of INH to INA is hydrolysis by an amidase. Further experiments were performed in rat hepatic microsomes to identify the oxidative step.

Fig. 3. Effect of Enzyme Inhibitors on INA Formation from INH in Isolated Hepatocytes from PB-Treated Rats
INH (0.5 mM) was incubated at 37°C in the presence of such enzyme inhibitors as metyrapone (0.02 mM) (△), cimetidine (0.5 mM) (▲) and bis-p-nitrophenylphosphate (BNPP) (0.1 mM) (□), respectively, in isolated hepatocytes (8×10⁶ cells/ml) from PB-treated rats. As a control group, INH was incubated in the absence of an enzyme (○). Each point represents the mean ± S.D. (n = 3) (* p < 0.01, ** p < 0.001).
In 1981, Muakkassah et al. suggested the possibility of an oxidative pathway in INH metabolism by recording the difference spectrum of INH with P450, but no direct oxidation of INH itself has yet been demonstrated. This time, we measured the difference spectra not only of INH but also of INAA with P450 in rat hepatic microsomes in order to compare the NADPH dependencies of these two compounds. After rat hepatic microsomal suspension of INH or INAA was divided into two cuvettes, a K-P buffer solution of INH or INAA was added to a sample cuvette, while the reference cuvette was added the K-P buffer solution only. The type II spectrum was initially observed just after the addition of INH or INAA. A marked spectral change was remarkable in INH after the addition of NADPH. By repetitive scanning every 2 min, the time-dependent changes of the spectrum in INH observed at 444 nm were found to be transitory (Fig. 4A). At 4 min after the addition of NADPH, a maximal broad peak appeared at around 490 nm, while INAA did not show an NADPH-dependent change in the spectra (Fig. 4B). The NADPH-dependent change of INH thus indicates the formation of the MI complexes of INH with P450, which supports the presence of possible oxidative cleavage in INH metabolism. The same spectral changes were also observed in benzoic acid, which includes benzene ring instead of pyridine ring of INH.

For P450-dependent oxidations, NADPH and oxygen are both essential. Therefore, the indispensability of both factors for INA formation was examined in the hepatic microsomal fraction using INH as a substrate. As shown in Fig. 5, INA formation took place aerobically in the presence of NADPH, and especially for the PB- and 3MC-treated groups, INA formation was significantly increased. Otherwise, the formation amount of INA decreased remarkably in the absence of either NADPH or oxygen in the untreated, PB- and 3MC-treated groups. We noticed by preliminary experiments that INA was formed from INH in the lymphoblast microsomes expressing CYP1A2, CYP2B6 and CYP3A4, which may provide strong evidence for P450-dependent oxidation of INH in humans.

As shown in Fig. 6, INA was produced P450-dependently from INH in untreated, PB- and 3MC-treated microsomes, respectively, while for AcINH and INAA, INA was little detected even in PB- and 3MC-treated microsomes. Regarding AcINH and INAA, occurrence of the oxidation on the nitrogen atoms, which is regarded as the first step of the oxidative formation of INA, might be difficult, since such an electronegative group as acetyl group or carboxyl group binds directly to the terminal nitrogen atom in INH or INAA. However, it is not yet clear whether or not P450-dependent oxidation of INH in humans.
ever, it is interesting that P450-dependent changes similar to those appearing in INH were also observed in benzoylhydrazine (unpublished data).

The following mechanism can thus be suggested to account for an oxidative cleavage of acid hydrazides (INH, benzoylhydrazine, etc.) to acids as follows:

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\text{Ar-CONHNH}_2 \rightarrow \text{Ar-CONHNHOH} \rightarrow \text{Ar-CON}=\text{NH} \rightarrow \text{Ar-CHO} \rightarrow \text{Ar-COOH}.
\]