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CYP2E1 mediated isoniazid-induced hepatotoxicity in rats

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KEY WORDS isoniazid; rifampin; hydrazines; cytochrome P-450 CYP2E1

ABSTRACT

AIM: To investigate the role of CYP2E1 in isoniazid (INH)-induced hepatotoxicity and the influence of rifampicin (RFP) on INH-induced liver injury. **METHODS:** Rats were treated with INH alone (100 mg/kg, ip) or co-administered with RFP (100 mg/kg, ig) for 10 d and 21 d. Hepatotoxicity was assayed by plasma enzymes (sALT, sAST) and histopathological examinations. Hepatic CYP2E1 activity was measured by aniline hydroxylase (ANH), and CYP2E1 mRNA expression was determined by RT-PCR. Plasma hydrazine concentration was determined by RP-HPLC. **RESULTS:** For a 10 d INH-treatment, hepatic CYP2E1 level was increased to 3.7-fold over the control; liver impairment appeared after 21 d treatment, while CYP2E1 and plasma hydrazine were, respectively, increased to 4.6-fold and 1.7-fold. However, in INH-RFP group for 10 d, CYP2E1 and plasma hydrazine were, respectively, decreased by 13 % and 18 % over INH group; similarly, hepatic injury is equal to INH group appeared after 21 d, and CYP2E1 was further decreased by 26 %. Correlation analysis showed that sALT had a positive correlation with plasma hydrazine and with CYP2E1 activity; CYP2E1 activity was also markedly correlated with plasma hydrazine. And compared with control, there is no difference in changes of CYP2E1 mRNA expression in INH and INH-RFP treatment for 21 d. **CONCLUSION:** The metabolite of INH, hydrazine, plays an important role in INH-induced hepatotoxicity in rats. The induction of CYP2E1 by hydrazine is involved in the hepatotoxicity of INH. RFP does not exacerbate INH-induced hepatotoxicity in short term, which relates to down-regulation of CYP2E1.

INTRODUCTION

Isoniazid (INH) in the treatment of all types of tuberculosis (TB) is associated with mild to moderate elevation of liver enzyme activity in plasma, and severe hepatotoxicity in approximate 1 %-2 % of patients. Acetylhydrazine, the metabolite of INH, has been suggested to be the cause of hepatic damage in patients. Recently, hydrazine, not INH or acetylhydrazine, has been reported to be most likely involved in the pathogenic mechanism of hepatic necrosis of INH-induced hepatotoxicity in rabbits[1]. Thus, though near 30 years after the toxicity was detected in INH-treated patient, the mechanism is still unknown[2]. Cytochrome P4502E1 (CYP2E1) is constitutively expressed in human liver and is responsible for the metabolic bioactivation of a wide variety of xenobiotics (including hepatotoxin CCl_4 and acetaminophen hepatotoxicity)[3,4], however its role in INH-induced hepatotoxicity is unclarified, as INH itself is an inducer of CYP2E1 [5].

Rifampicin (RFP) is usually co-used with INH to treat TB. The combination of INH and RFP was reported to result in a higher rate of inhibition of biliary secretion and an increase in liver cell lipid peroxidation, and cytochrome P450 was thought to be involved in the synergistic effect of RFP on INH[6]. Meanwhile, recent

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studies suggest that RFP suppresses the expression of CYP2E1 and has the protective effect on CCl_4-induced hepatotoxicity by reducing the formation of free radicals[7,8].

Human hepatic CYP2E1 is homologous to that of rats[9]. Thus, in present study, we investigate the role of CYP2E1 in INH-induced hepatotoxicity and the influence of RFP on the hepatotoxicity of INH in rats.

MATERIALS AND METHODS

Materials INH was from Sanjiu Wanrong Pharmaceutical Co, RFP was from Sichuan Pharmaceutical Co. Isocitric acid, isocitric dehydrogenase, NADP, 3-meth-oxybenzaldehyde (MBA), 9-fluorenone and hydrazine were purchased from Sigma Chemical Co (USA), erythromycin was from AMSERCO (USA). ALT kit and AST kit were from Shanghai Rongsheng Biotechnology Co and Shanghai Biologic Products Institute, respectively. TRIzol reagent, Gibco Co; TaKaRa one step RNA PCR kit (AMV), TaKaRa Biotechnology Co. Primers of CYP2E1 and cyclophilin (CYC) were custom synthesized by Gibco Co. Methanol and acetonitrile of HPLC-grade, Fisher Co, and all the other chemicals and reagents were of analytical grade.

Animals and treatment Male Wistar rats (152±6 g, *n*=36, Certificate No 19-084) were supplied by Experimental Animals Center, Hubei Province. Throughout the study, rats were housed in temperature-controlled rooms with 12-h light-dark cycle and were given free access to food and water. Rats were treated with INH (100 mg/kg, ip) alone or co-administered with RFP (100 mg/kg, ig, in sterile water) for 10 d and 21 d. Untreated rats were used as the control. They were sacrificed 1 h after administration on d 10 or d 21, respectively.

Biochemical analysis Activities of sALT and sAST were determined using test kits. Liver microsomes were prepared by differential ultra-centrifugation. Aniline hydroxylase (ANH) activity, as the marker of CYP2E1, was assayed as previously described[10]. Liver microsomes (protein 1 mg) were incubated with aniline 0.8 mmol/L and NADPH-generating system (containing isocitric acid 11.7 mmol/L, NADP 0.47 mmol/L, isocitric acid dehydrogenase 0.7 U) at 37 ºC for 30 min. Protein was assayed by the method of Lowry[11].

Plasma hydrazine determination Plasma hydrazine concentration was determined by RP-HPLC[1,12]. The solvent delivery system was a Shimadzu pump model LC-9A. The analytical column was Zorbax SB-C18 (4.6 mm ID×25 cm, 5 μm particle size), and column effluent was monitored with SPD-6AV ultraviolet spectrophotometric detector at 300 nm. The mobile phase was sodium acetate 5 mmol/L (pH 5.0)-CH_3CN (35:65, v/v). Samples were denatured by propanol, then reacted with derivatizing reagent that consisted of MBA 8.22 mmol/L, 9-fluorenone 2 mmol/L and formic acid 15 % (v/v). The calibration curve of hydrazine concentration (C) *vs* the ratio for area (Y) of hydrazine to 9-fluorenone results in a correlation coefficient (r) of 0.999. And the linear range for hydrazine in plasma was from 8.5 to 85.2 μmol/L. The regression equation was Y=0.057 C+1.577. The average recovery rate was 100.2 %, and the relative standard deviation of intra-day and inter-day were all less than 2 % (Fig 1, Tab 1).

Hepatic CYP2E1 mRNA expression Total RNA was extracted from liver tissue using TRIzol reagent and stored at -80 ºC until used. Primers of CYP2E1 and

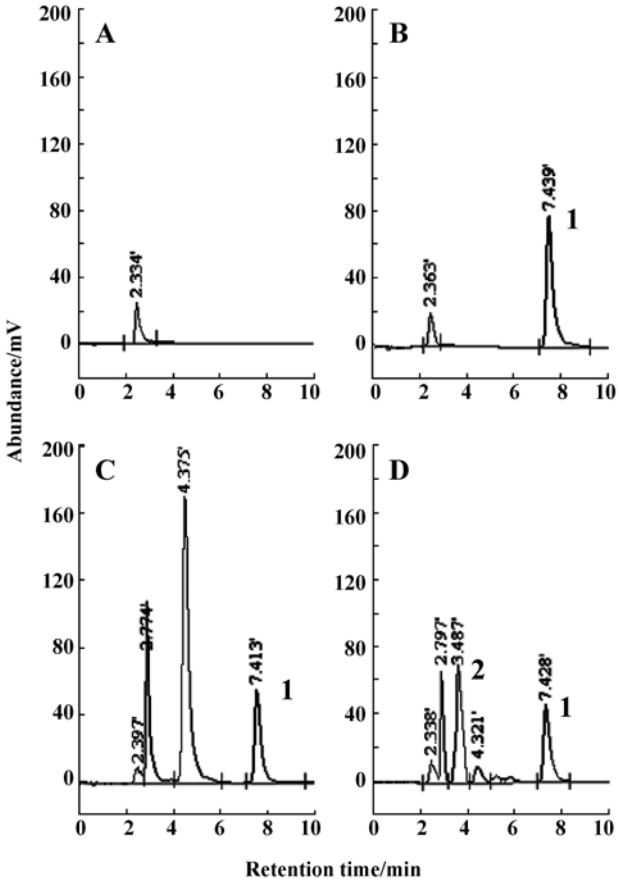

Fig 1. RP-HPLC chromatograms of plasma hydrazine. A) blank plasma, B) blank plasma spiked with internal standard, C) blank plasma spiked with derivatizing reagent, D) sample reacted with derivatizing reagent for 3 h. Peaks: 1, 9-fluorenone (internal standard); 2, hydrazine (derived product).

Tab 1. Recovery of hydrazine in plasma. *n*=5. Mean±SD.

Spiked amount/ μmol·L^{-1}	Measured amount/ μmol·L^{-1}	Recovery/%	Reproducibility/%	
			Intra-day	Inter-day
8.5	8.3±0.4	97±5	0.03	0.27
17.1	17.2±1.0	101±6	0.14	1.16
51.1	52.5±1.0	103±2	0.07	0.06

interior standard (cyclophilin, CYC) were designed as previously described[13]. The expected lengths of PCR products were 473 bp (CYP2E1) and 265 bp (CYC). The reaction for conversion of total RNA to cDNA was carried out with TaKaRa AMV in AMPLITRON II thermolyne at 42 ºC for 1 h. PCR reaction was carried out with TaKaRa *Taq* for 29 cycles, and amplified cDNA products were separated by 2 % agarose gels with ethidium bromide. The images were taken by Kodak digital camera and the relative intensities of CYP2E1 to CYC were analyzed with ID image analysis software.

Statistical analysis Data were presented as mean±SD and analyzed by two-tailed *t*-test. A probability level less than 0.05 was considered significance. Correlation was analyzed with linear regression.

RESULTS

Effects of INH/INH-RFP treatment on the level of sALT, sAST and CYP2E1 For 10 d INH-treatment, sALT, sAST and liver index were not markedly changed. However, after 21 d INH-treatment, sALT and liver index were, respectively, increased by 31 % and 21 % over the control (P<0.05), which indicated the development of liver damage. Hepatocellular disintegrate and the vacuolation in the liver was observed in the centrilobular region by histopathological examinations. Meanwhile, hepatic CYP2E1 level was increased to 3.7-fold and further increased to 4.6-fold, respectively, for 10 d (P<0.05) and 21 d INH-treatment (P<0.01), which expressed the induction of CYP2E1 by INH (Tab 2, Fig 2).

Liver functions were not significantly changed in rats co-treated with INH and RFP for 10 d; similarly, sALT was not further increased in INH-RFP group when hepatotoxicity appeared after 21 d INH-treatment, and the slices exhibited equal degree of hepatic injury in these two groups. The results indicated RFP did not obviously exacerbate liver impairment induced by INH. In addition, CYP2E1 level in INH-RFP group was decreased by 13 % after 10 d treatment (P<0.05) and further decreased by 26 % after 21 d treatment *vs* INH group, respectively (Tab 2, Fig 2).

Effects of INH / INH-RFP treatment on plasma concentration of hydrazine Plasma hydrazine concentration is 31 μmol/L in rats treated with INH for 10 d, and it was increased to 1.7-fold at d 21 (P<0.01). Plasma hydrazine in rats co-treated with INH and RFP was decreased by 18 % over INH group at d 10 (P< 0.05), and it was at the similar level of INH group at d 21 (Fig 3).

Correlation analysis To determine whether the changes of sALT, plasma concentration of hydrazine, and liver microsomal CYP2E1 levels were related, several correlation analysis were calculated. There is a significant correlation between sALT activity and plasma concentration of hydrazine in rats in INH group (r=0.696, P<0.05, Fig 4), supporting that the metabolite of INH, hydrazine, is involved in INH-induced hepatotoxicity. Furthermore, hepatic CYP2E1 activity was correlated markedly with plasma hydrazine (r=0.626, P<0.05, Fig 5). And there was also a significantly positive correlation between sALT and CYP2E1 (r=0. 904, P<0.01, Fig 6).

Effects of INH on rat hepatic CYP2E1 mRNA expression Compared with control, there was no

Tab 2. Effects of isoniazid and rifampicin treatment for 10 d and 21 d on sALT, sAST and CYP2E1 level in rats. *n*=6. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* control. ^{e}P<0.05, ^{f}P<0.01 *vs* isoniazid group.

Treatment	10 d				21 d			
	sALT/ U·L^{-1}	sAST/ U·L^{-1}	ANH/ μmol·min^{-1}·g^{-1}	Liver index %	sALT/ U·L^{-1}	sAST/ U·L^{-1}	ANH/ μmol·min^{-1}·g^{-1}	Liver index/ %
Control	10±5	60±14	0.56±0.03	3.47±0.15	32±7	124±14	0.73±0.10	3.30±0.19
INH	11±8	65±9	2.08±0.15^{e}	3.48±0.12	42±6^{b}	135±20	3.37±0.28^{c}	3.99±0.20^{e}
INH-RFP	10±6	53±9	1.81±0.21^{ce}	3.47±0.07	43±10^{b}	140±8^{b}	2.5±0.4^{cf}	4.00±0.19^{e}

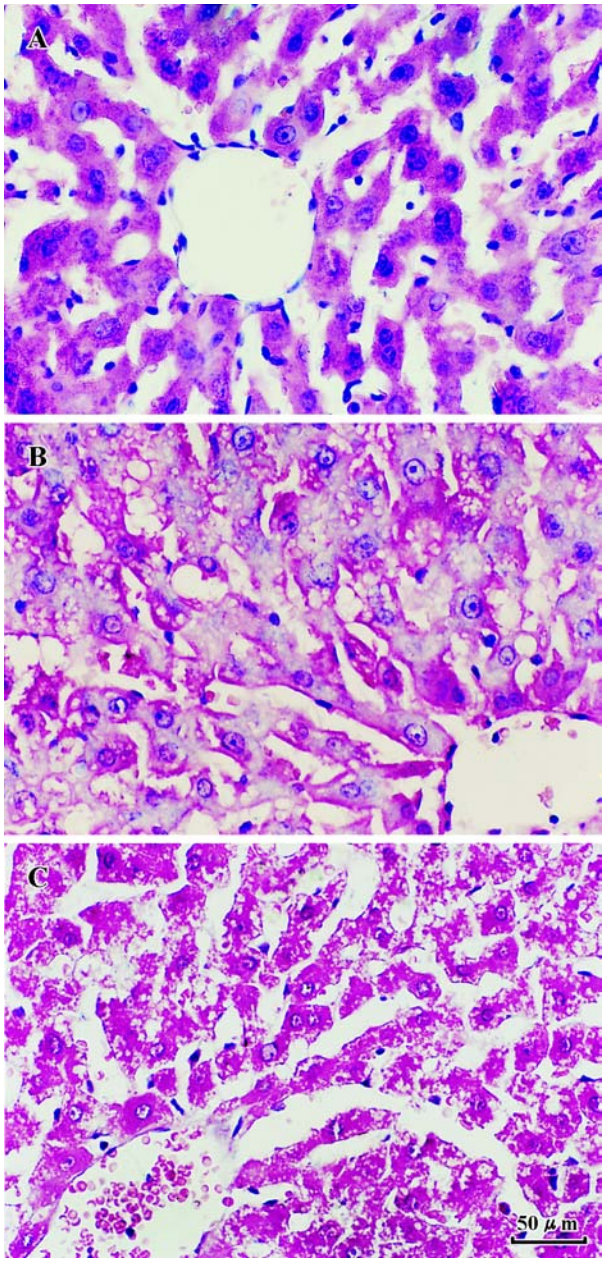

Fig 2. Histopathological examinations of the liver in rats treated for 21 d. A) control; B) isoniazid group; C) isoniazid-rifampicin group. (HE staining, ×400).

significant difference of CYP2E1 mRNA expression between INH and INH-RFP treatment for 21 d in rats (Fig 7).

DISCUSSION

During the metabolism of INH, hydrazine is produced directly (from INH) or indirectly (from acetylhydrazine). From our study, it is evident that hydrazine plays a role in INH-induced liver damage in rats,

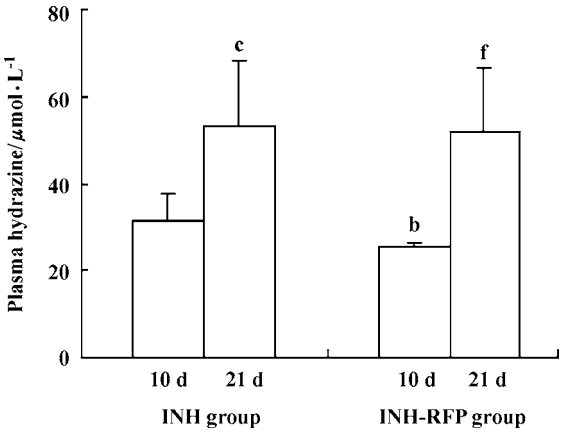

Fig 3. Effects of isoniazid and rafampicin treatment for 10 d and 21 d on plasma concentrations of hydrazine in rats. n=6. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* isoniazid group for 10 d. ^{f}P<0.01 *vs* isoniazid-rifampicin group for 10 d.

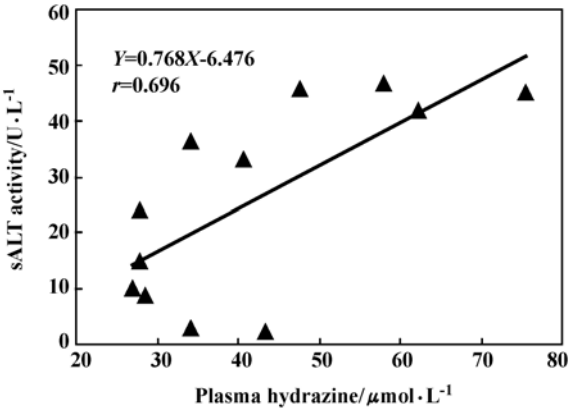

Fig 4. Correlation of sALT activity with plasma hydrazine concentration in rats treated with isoniazid (P<0.05, n=12).

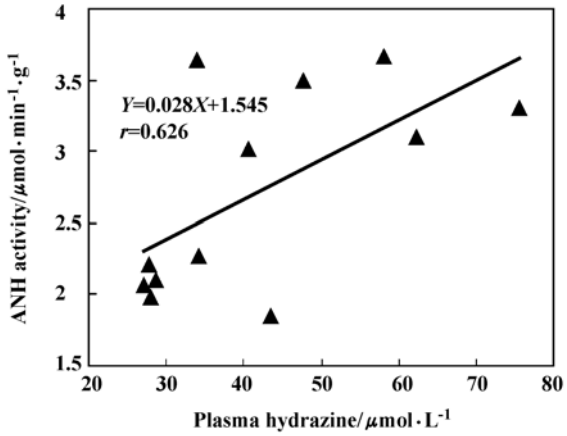

Fig 5. Correlation of hepatic ANH (CYP2E1) activity with plasma hydrazine concentration in rats treated with isoniazid (P<0.05, n=12).

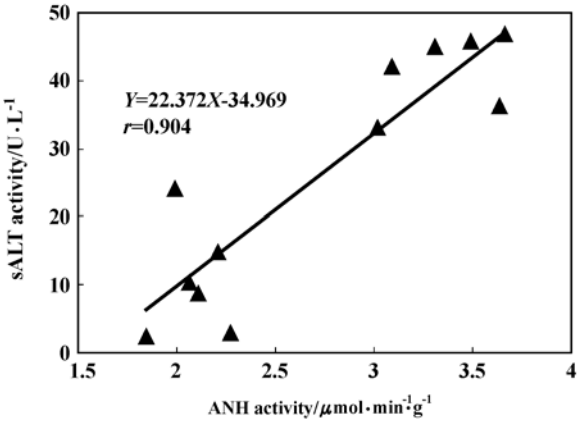

Fig 6. Correlation of sALT activity with hepatic ANH (CYP2E1) activity in rats treated with isoniazid (P<0.01, n=12).

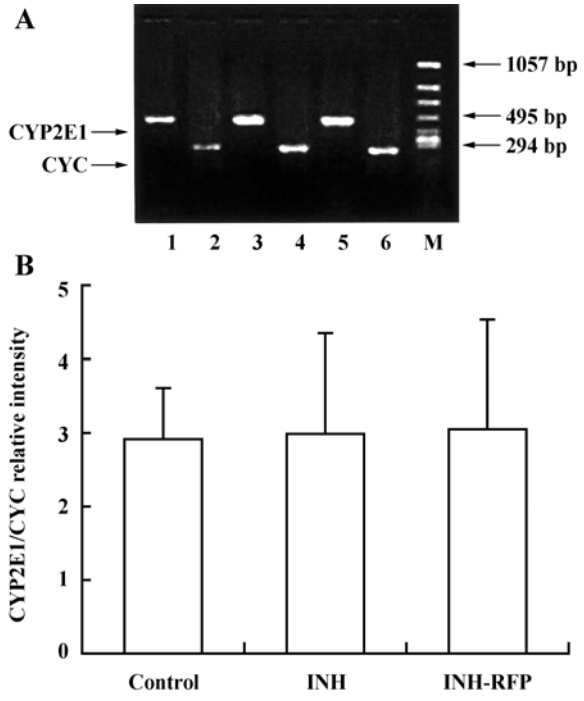

Fig 7. Effects of isoniazid and rifampicin treatment for 21 d on hepatic CYP2E1 mRNA expression in rats. A) Amplified products of CYP2E1 and CYC were separated by 2 % agarose gels. Lane 1, 2: control; Lane 3, 4: isoniazid group; Lane 5, 6: isoniazid-rifampicin group; Lane M: marker. B) The relative intensities of CYP2E1 to CYC were analyzed. n=3. Mean±SD.

which is consistent with the report by Sarich *et al*[1] who found the severity of INH-induced hepatocellular damage had a positive correlation with plasma hydrazine levels in rabbits.

The main positive finding of the present study is the significant correlation between the marker of CYP2E1, aniline hydroxylase, and plasma hydrazine concentration, which suggests that the induction of CYP2E1 by INH may be mediated by its metabolite, hydrazine. Powell *et al*[14] investigated the variability in the level of CYP2E1 mRNA, protein and functional activity, and found that the variation in CYP2E1 mRNA (18-fold) was greater than the variation seen CYP2E1 protein (2-fold) and functional activity (4-fold), suggesting there was no correlation between functional activity and mRNA level. This may explain our observation, that CYP2E1 activity was up-regulated by INH, while CYP2E1 mRNA expression level showed no change.

Administration of INH to rats leads to increase in the plasma concentration of hydrazine and CYP2E1 activity, and when both of them were generated to a certain level, hepatotoxicity occurred. It is known that the best role of CYP2E1 is the production of free radicals[15], we speculate that CYP2E1 mediated the hydrazine-induced liver damage may relate to this characteristic. This speculation is supported by the observation, that the combination of RFP and INH in short term (10 d and 21d) does not further exacerbate INH-induced hepatotoxicity accompanying with the down-regulation of CYP2E1 activity, and by the fact that RFP protects CCl_4-induced liver injury by suppressing CYP2E1 expression[7,8].

In conclusion, the induction of CYP2E1 by hydrazine is involved in INH-hepatotoxicity, and RFP does not exacerbate hepatotoxicity of INH in short-term via the down-regulation of CYP2E1. Thus, these results are valuable to understand the possible mechanism of INH-hepatotoxicity and further to predict the interaction of INH and RFP in clinic.

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