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HYDRAZINE RADICAL FORMATION CATALYZED BY RAT MICROSOMAL NADPH-CYTOCHROME P-450 REDUCTASE

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SUMMARY: Using NADPH-cytochrome P-450 reductase purified from rat liver microsomes, the oxidation of hydrazine to its radical was proved to proceed smoothly. The catalytic effect of NADPH-cytochrome P-450 on the radical formation in the hepatic microsomes obtained from phenobarbital-pretreated rats was also supported by the fact that Hz radical formation was stimulated by flavin adenin dinucleotide or methyl viologen and markedly inhibited by superoxide dismutase, however, carbon monoxide showed no effect. Expectedly, anti-NADPH-cytochrome P-450 IgG decreased the radical formation. The present study provides the first evidence for the NADPH-cytochrome P-450 reductase catalyzed oxidation of hydrazine to its radical in the presence of O_2 and NADPH.

It is well-known that isoniazid (INH)-induced hepatic injury is markedly enhanced during about 2 weeks after INH-rifampicin (RMP) coadministration to the patients (1,2). A series of our studies on irreversible hepatocellular damage during INH therapy suggested strongly that the formation of active metabolic intermediates of hydrazine (Hz), a hydrolyzed metabolite of INH, such as a radical or diimide during metabolism probably plays an important role in the hepatotoxic, mutagenic and carcinogenic processes mediated by INH (3-8). Recently, we succeeded in isolating a radical adduct with α-phenyl-t-butylnitrone (PBN) during oxidative metabolism of Hz in rat liver microsomes

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Abbreviation: FAD, flavin adenin dinucleotide; fp_2, NADPH-cytochrome P-450 reductase; Hz, hydrazine; INH, isoniazid; MV, methyl viologen; PB, phenobarbital; PBN, α-phenyl-t-butylnitrone; RMP, rifampicin; SOD, superoxide dismutase.

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(9). The spin-trapped species was identified as the Hz radical by comparing its ESR parameters and mass spectrum with those of the adduct formed during the chemical oxidation of Hz catalyzed by cupric chloride. The requirement of oxygen and NADPH in the microsomal oxidation and the occurrence of a typical binding spectrum by difference spectroscopy seemed to suggest the participation of the cytochrome P-450 system in the formation of the Hz radical which must be a precursor of diimide during microsomal oxidation of Hz (9). The present communication, however, provides the first evidence for in vitro oxidation of Hz to Hz radical catalyzed by NADPH-cytochrome P-450 reductase (fp_2).

Materials and Methods

Chemicals
Hz sulfate and NADPH were purchased from Tokyo Ind. Co. Ltd. and Oriental Yeast Co. Ltd. Japan, respectively. All other chemicals were of analytical or reagent grade.

Animal experiments and enzyme evaluation
Liver microsomal fraction of male Wistar rats pretreated with phenobarbital (PB) sodium or RMP was prepared by the method previously reported (4). Both measurements of cytochrome P-450 contents (10) and fp_2 activity (11) and the purification of fp_2 from the hepatic microsomal fraction (12,13) were performed by the methods as already reported.

Anti-fp_2 IgG separation
Anti-fp_2 IgG was prepared followed by separation from the rabbit serum by using a modification of the method of Sumimoto et al. (14).

Enzyme reaction and analytical method
The experimental conditions of fp_2 system are shown in the legends of Fig. 1 and 2, respectively, in detail. In general, the enzyme solution containing a substrate and NADPH was incubated at 37°C using PBN as a radical trapping agent. The reaction mixture was extracted with benzene and the layer was dried over anhydrous sodium sulfate. After evaporation, the residue was chromatographed with ether using thin layer silica gel plate. An ESR signal was detected from the region around Rf value at 0.5, which includes PBN-adduct of Hz radical. The ESR and mass spectra of the $CHCl_3$ extract were obtained by the same instruments as previously reported (9). The amount of Hz radical was estimated from the peak height ratio of PBN-adduct of Hz radical (M^+, m/z 208) to that of antipyrine (M^+, m/z 188, internal standard).

Results

As was reported in our previous paper, the Hz radical formation was demonstrated as a PBN-adduct by means of both ESR spectroscopy and mass spectrometry (9). In order to elucidate the mechanism of the radical formation, the

effect of carbon monoxide (CO) gas was examined in the microsomal system. However, PBN-adduct of Hz radical was isolated without an apparent depression in the yield, from which the prominent participation of cytochrome P-450 dependent monooxygenase system on the oxidation of Hz to its radical was completely excluded. Since alkylhydrazines can be oxidized by flavine containing monooxygenase (15,16), an attempt was made to inhibit the reaction by using methimazole, which is well-known as an inhibitor of this monooxigenase. However, no inhibition was observed.

Tatsumi et al. reported that fp_2 appeared to play a prominent role in the oxidation of 1,1-diphenylhydrazine to the corresponding N-nitrosamine (17). Therefore, the effects of flavin adenin dinucleotide (FAD), methyl viologen (MV) or superoxide dismutase (SOD) on the reaction were examined in PB-pretreated rat liver microsomes. The Hz radical formation was remarkably accelerated by the addition of FAD and MV to 250 and 200% of the control value, respectively, while the radical amount was markedly decreased to 25% of the control value by SOD addition.

The results mentioned above seems to reveal a strong possibility that one electron oxidation of the Hz to Hz-radical is fp_2 dependent. In order to prove this idea, the reaction was performed in the purified fp_2 enzyme system supplemented with NADPH. The Hz radical was, as expected, able to be detected in the reaction mixture as a PBN-adduct (Fig. 1 and 2), and the amount was reduced again by SOD addition to 39 % of the control value. Furthermore, by adding rat anti-fp_2 IgG the radical formation was inhibited to 75 % of the control value.

From these facts mentioned above, it is apparent that we have found the first evidence for the fp_2-catalyzed oxidation of Hz to the Hz-radical.

Discussion

In vitro biotransformation of 1,1-diphenylhydrazine to N-nitrosodiphenylamine reported by Tatsumi et al. is one of the few examples of fp_2 catalyzed oxidation (12). The present study, however, gives the first evidence for the fp_2 catalyzed one electron elimination from the Hz molecule, which is the

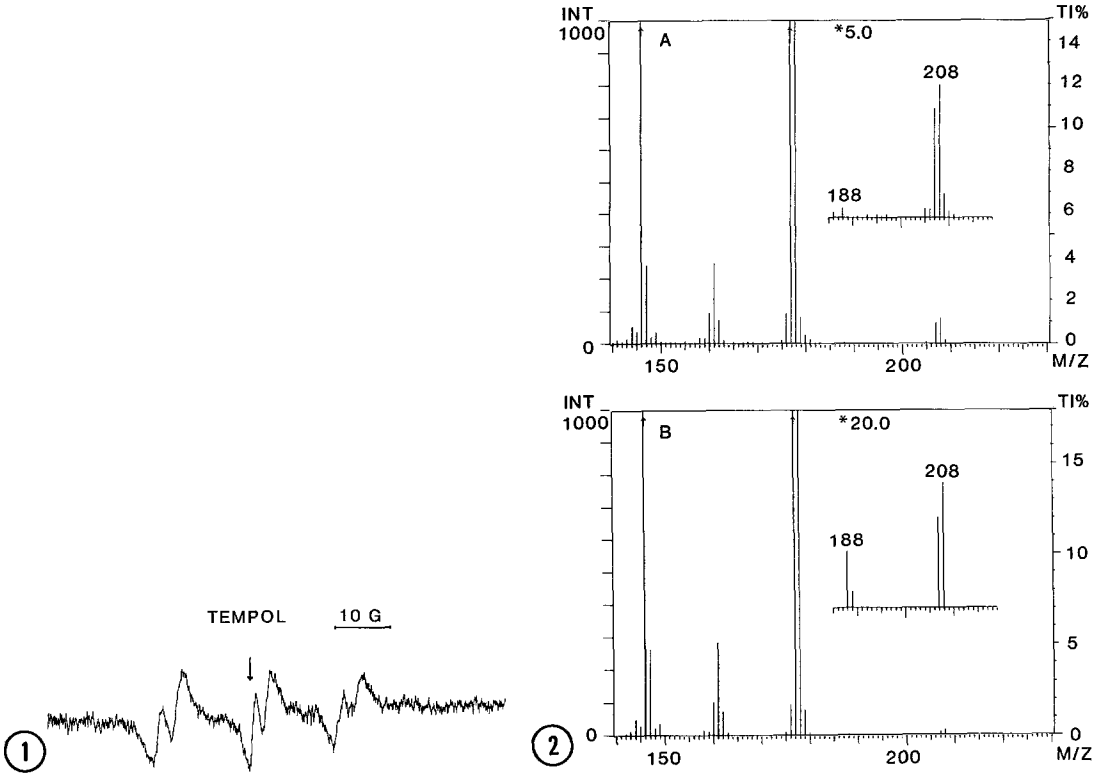

Fig. 1 ESR spectrum of PBN-adduct of hydrazine radical produced in the purified fp_2 system. The adduct was obtained from benzene extract after the incubation using microsomal fp_2 purified from rat liver. The reaction mixture consisted of 5.6 ml of fp_2 suspension (2.4 unit), 2.0 ml of PBN solution (24 mM), 0.2 ml of hydrazine solution (4.8 mM) and 0.2 ml of NADPH solution (1.0 mM). The solution was prepared by using 1/15 M potassium phosphate buffer solution (pH 7.4). The incubation was performed at 37°C for 30 min under an aerobic condition. The spectrum was measured in chloroform by using an extract after TLC purification. ESR conditions: microwave power, 3 mW; modulation amplitude, 1G; sample temperature, 22.5°C. TEMPOL: 2,2,6,6-tetramethyl-4-hydroxypiperidyl 1-oxyl.

Fig. 2 The effect of anti-fp_2 IgG on hydrazine radical formation in the purified fp_2 system. The enzyme inhibition was evaluated from the intensity ratio between the PBN-adduct (M^+, 208) and antipyrine (M^+, 188). The adduct was obtained from benzene extract after the incubation using microsomal fp_2 purified from rat liver in the absence (A) or presence (B) of anti-fp_2 IgG. The sample (A) is the same one used in ESR experiments as shown in the legend of Fig. 1. The enzyme solution (B) contains both fp_2 and the equi-amount of anti-fp_2 IgG. The addition of anti-fp_2 IgG inhibited the radical formation, while nonimmune IgG did not give any effect on the reaction.

first step in the conversion of Hz to its radical, in the presence of molecular oxygen and NADPH. During the course of the oxidation of Hz to the ultimate metabolite, nitrogen gas (18), the formation of various intermediates by the participation of microsomal enzymes other than fp_2 are, of course, probable. Indeed, our previous report described a contribution of cytochrome P-450

to the oxidation of Hz, which was clarified by the estimation of a difference spectrum of cytochrome P-450 (9). In this report we have not presented the detailed data which could possibly reveal the precise role of cytochrome P-450 in the oxidation of Hz. However, our preliminary experiments suggest that the cytochrome P-450 probably contributes to the metabolic conversion of the Hz radical to diimide, because metyrapone or PBN addition to PB-pretreated rat liver microsomes inhibited the maximum peak of a different spectrum between diimide and cytochrome P-450. The inhibition of $\Delta Ab_{448-490}$, 3 min after the addition of 0.02 or 5 mM of each inhibitor, were 14.7% and 31.9% of the control value, respectively. Furthermore, it is also assumed from the preliminary in vivo experimental data, in which RMP pretreatment (i.p., 30 mg/kg for six days) accelerates the fp_2 activity (126% and 111% of the control value) 1 and 3 hr after Hz administration (i.p., 0.8 mmol/kg) to rats. Further studies are in progress to establish the roles of enzymes and RMP which participate in Hz oxidation and INH-induced hepatic injury.

References

1. Pessayre,D. (1982) Agressologie 23, 13-15.
2. Tsagaropoulou-Stinga,H. et al. (1985) Pediatric Infectious Disease 4, 270-273.
3. Noda,A., Hsu,K-Y., Noda,H., Yamamoto,Y. and Kurozumi,T. (1983) J.UOEH (Sangyo-Idai Zasshi) 5, 183-190.
4. Sendo,T., Noda,A., Noda,H., Hsu,K-Y. and Yamamoto,Y. (1984) J.UOEH (Sangyo-Idai Zasshi) 6, 249-255.
5. Noda,A., Sendo,T., Ohno,K., Goto,S., Noda,H. and Hsu,K-Y (1985) Toxicol. Lett. 25, 313-317.
6. Noda,A., Sendo,T., Ohno,K., Misaka,A., Kanazawa,Y., Isobe,R., Goto,S., Ishizawa,M., Noda,H. and Hirata,M. (1986) J.Pharm.Dyn. 9, s-45.
7. Noda,A., Ishizawa,M., Ohno,K., Sendo,T. and Noda,H. (1986) Toxicol.Lett. 31, 131-137.
8. Noda,A., Sendo,T., Ohno,K., Noda,H. and Goto,S. (1987) Chem.Pharm.Bull. 35, 2538-2544.
9. Noda,A., Noda,H., Ohno,K., Sendo,T., Misaka,A., Kanazawa,Y., Isobe,R. and Hirata,M. (1985) Biochem.Biophys.Res.Commun. 133, 1086-1091.
10. Matsubara,T., Koike,M., Touchi,A., Tochino,Y. and Sugeno,K. (1976) Anal. Biochem. 75, 596-603.
11. Omura,T. and Takesue,S. (1970) J.Biochem. 67, 249-257.
12. Yasukochi,Y. and Masters,B.S.S. (1976) J.Biol.Chem. 251, 5337-5344.
13. Sakai,H., Hino,Y. and Minakami,S. (1983) Biochem.J. 215, 83-89.
14. Sumimoto,H., Takeshige,K and Minakami,S. (1988) Eur.J.Biochem. in press.
15. Wittkop,J.A., Prough,R.A. and Reed,D.J. (1969) Arch.Biochem.Biophys. 134, 308-315.
16. Prough,R.A., Freeman,P.C. and Hines,R.N. (1981) J.Biol.Chem. 256, 4178-4184, and references cited therein.
17. Tatsumi,K., Kitamura,S. and Sumida,M. (1984) Biochem.Biophys.Res.Commun. 118, 958-963.
18. Nelson,S.D. and Gordon,W.P. (1981) Adv.Exper.Med. 136B, 971-981.