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METABOLIC ACTIVATION OF TRP-P-2, A TRYPTOPHAN-PYROLYSIS MUTAGEN BY ISOLATED RAT HEPATOCYTES

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Abstract—Metabolic activation of a tryptophan-pyrolysis product, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), by isolated rat hepatocytes was studied. The substrate (Trp-P-2) disappearance by hepatocytes from untreated rats was slow, but enhanced by 3-methylcholanthrene (MC) pretreatment of rats. The covalent binding of Trp-P-2 to cellular macromolecules was detected in hepatocytes from untreated rats. The amount of covalent binding of Trp-P-2 to protein and RNA was greater than that to DNA. The covalent binding to Trp-P-2 to DNA, RNA and protein in hepatocytes from untreated rats was about 5–10 times less than that in hepatocytes from MC-pretreated rats. 7,8-Benzoflavone strongly inhibited the substrate disappearance and the binding of Trp-P-2 to DNA in hepatocytes from MC-pretreated rats. These results indicate that Trp-P-2 is metabolically activated by the P-448 type of cytochrome P-450 which is induced by MC. Diethylmaleate enhanced by about 50% the binding of Trp-P-2 to DNA in hepatocytes from MC-pretreated rats. On the other hand, cysteine inhibited the binding of Trp-P-2 to DNA with a concomitant reduction in the accumulation of the active metabolite, *N*-hydroxy-Trp-P-2 (*N*-OH-Trp-P-2). Sulfhydryl compounds seemed to play important roles in the detoxification of Trp-P-2.

Recently, several promutagens have been found in pyrolysates of protein and amino acids [1, 2]. Trp-P-2, which is formed by pyrolysis of tryptophan, shows a potent mutagenic activity toward *Salmonella typhimurium* [3, 4]. Trp-P-2 has also been demonstrated to be carcinogenic to rats [5] and mice [6]. Since this compound was found originally in cooked food [7], Trp-P-2 may be significant as an environmental carcinogen. Therefore, it is important to reveal the mechanism of metabolic activation and detoxification of Trp-P-2.

Trp-P-2, as well as many other chemical carcinogens, requires metabolic activation to exert its mutagenic and carcinogenic properties. It seems to be established that interactions of activated carcinogens with DNA are involved in the initiation of carcinogenesis. It has been demonstrated that Trp-P-2 binds covalently to DNA *in vitro* [8, 9]. However, the metabolic activation and covalent binding of Trp-P-2 *in vivo* have remained obscure.

In order to estimate the metabolic activation of xenobiotics *in vivo*, a system of isolated hepatocytes offers a useful model. The metabolism of xenobiotics by isolated hepatocytes is thought to closely resemble the *in vivo* situation. Thus far, metabolisms of several carcinogens have been studied with isolated hepatocytes [10–17], and these studies have greatly contributed to our understanding of the metabolisms of carcinogens. In this paper, the metabolic activation of Trp-P-2 by isolated adult rat hepatocytes is described. Several novel aspects of the metabolic activation of Trp-P-2 could be demonstrated with isolated hepatocytes.

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MATERIALS AND METHODS

Materials. Trp-P-2 was supplied under the Research Resources Program for Cancer Research of the Ministry of Education, Science and Culture of Japan. [^{3}H]Trp-P-2 (344 mCi/mmole) was prepared by New England Nuclear (Boston, MA). Collagenase (EC 3.4.24.3), ribonuclease (EC 3.1.4.22) and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Diethylmaleate (DEM), 7,8-benzoflavone (BF) and cysteine were from Wako Chemical Co., Japan, and 3-methylcholanthrene (MC) from Spectrum Chemicals, CA. Pronase was purchased from Kaken Kagaku Co., Japan. All other chemicals were of analytical grade.

Isolation of hepatocytes. Male Sprague–Dawley rats (150–250 g) were used. The treatment group of rats was given 15 mg of MC (0.5% solution in corn oil) per kilogram body wt intraperitoneally once daily for 3 days and killed 40 hr after the last injection. Hepatocytes were isolated by the perfusion method of Berry and Friend [18] with some modifications; briefly, livers were perfused *in situ* with Ca^{2+}, Mg^{2+}-free Hanks' buffer with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5) containing 0.5 mM ethyleneglycolbis(β-aminoethylether)*N,N'*-tetraacetic acid and followed by perfusion with the same Hanks' buffer containing 5 mM $CaCl_2$ and 0.1% collagenase. The separated hepatocytes were filtered through a nylon mesh and collected by centrifugation at 50 *g* for 1 min. The cells were washed twice with the same buffer. More than 85% of the cells were intact as judged by trypan blue exclusion.

Binding of Trp-P-2 to macromolecules of hepatocytes. Freshly isolated hepatocytes (2.5×10^6 cells/ml) were incubated with [^{3}H]Trp-P-

2 (30 μM unless otherwise stated) in Hanks' buffer with 10 mM HEPES (pH 7.4) containing 2% bovine serum albumin at 37° under an atmosphere of 95% O_2–5% CO_2. At indicated times, a 6-ml portion of the mixture was taken and DNA, RNA and protein were isolated, respectively, by the method of Kuroki and Heidelberger [19], except that the treatment of partially purified DNA with ribonuclease was followed by treatment with pronase (40 μg/ml) at 37° for 30 min. The radioactivity of each macromolecule was determined with liquid scintillation counting as described previously [9]. The amounts of DNA and RNA were measured by the diphenylamine method [20] and the orcinol method [21], respectively. Protein was determined by the method of Lowry *et al.* [22].

Analysis of Trp-P-2 metabolism. Unlabelled Trp-P-2 (30 μM) was added to the suspension of hepatocytes in the same Hanks' buffer as described earlier. At the indicated times, a 0.8-ml portion was taken and added to 0.2 ml of 1 M sodium acetate (pH 4.5) containing 5 M NaCl and 5 mM dithiothreitol. Acetonitrile (1 ml) was added to the mixture and the resulting mixture was centrifuged at 10,000 *g* for 20 min. The supernatant (50 μl) was applied to a high-performance liquid chromatograph (HPLC) equipped with a column of μBondapak C_{18}. The mobile phase was acetonitrile–20 mM sodium acetate (pH 4.5) [40:60 (v/v)]. Amounts of Trp-P-2 and *N*-OH-Trp-P-2 were determined from the peak heights measured at 254 nm, respectively, as described previously [9, 23]. When BF was added to the suspension of hepatocytes, [^{3}H]Trp-P-2 (17.1 mCi/mmole) was used instead of unlabeled Trp-P-2. The amounts of Trp-P-2 and *N*-OH-Trp-P-2 were determined with their radioactivities, since BF interfered with HPLC analysis of Trp-P-2.

RESULTS

The effect of MC pretreatment on the metabolism of Trp-P-2 was examined. Fig. 1 shows the decrease in unmetabolized Trp-P-2 with incubation time. About 90% of Trp-P-2 was metabolized within 30 min by hepatocytes from MC-pretreated rats, whereas more than 80% of Trp-P-2 remained unchanged with hepatocytes from untreated rats. Metabolism of Trp-P-2 was also strongly inhibited by BF with MC-pretreated rat hepatocytes. As shown in Fig. 2, Trp-P-2 was bound to each macromolecule linearly with incubation time up to 30 min. The amount of Trp-P-2 covalently bound to the DNA of the MC-pretreated rat hepatocytes was about 10 times more than that bound to the DNA of untreated rat hepatocytes. The same effect of MC pretreatment was observed on the covalent binding of Trp-P-2 to RNA and protein. BF decreased more than 90% of the amount of Trp-P-2 bound to DNA in the MC-pretreated rat hepatocytes (Table 1). These results indicate that Trp-P-2 is activated by a BF-sensitive cytochrome P-450(s) induced by MC.

It is well known that conjugation reactions play important roles in detoxification of many xenobiotics. Conjugation with sulfhydryl compounds is conceivably involved in the detoxification of Trp-P-2. DEM reacts with the sulfhydryl group and decreases

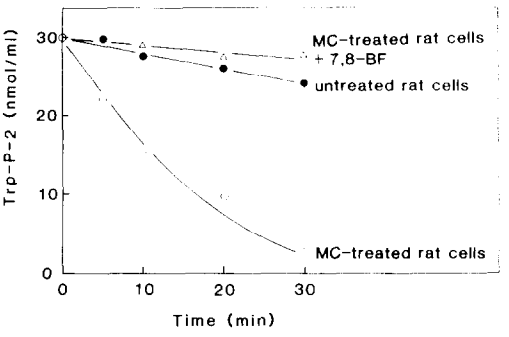

Fig. 1. Metabolism of Trp-P-2 by untreated or MC-pretreated rat hepatocytes. Concn of 7,8-BF was 0.5 mM.

the content of sulfhydryl compounds. Consequently, it inhibits the conjugation of electrophilic compounds with glutathione or cysteine. We examined the effect of DEM on the covalent binding of Trp-P-2 to macromolecules of hepatocytes. As shown in Table 1, DEM caused an increment of about 50% in the binding of Trp-P-2 to DNA of hepatocytes from MC-pretreated rats. DEM also increased the binding of Trp-P-2 to protein and RNA (data not shown). On the contrary, the binding of Trp-P-2 to DNA, RNA and protein was inhibited dose-dependently by cysteine (Table 2). These results suggest that sulfhydryl compounds are important in the detoxification of Trp-P-2.

It has been demonstrated with *in vitro* experiments that *N*-hydroxylation is an activation process of Trp-P-2 [23]. Fig. 3 shows the formation of *N*-OH-Trp-P-2 by hepatocytes. The accumulation of *N*-OH-Trp-P-2 was inhibited by the addition of cysteine. This effect of cysteine seemed attributable to the stimulation of the detoxification of *N*-OH-Trp-P-2. However, we cannot exclude the possibility that the formation of *N*-OH-Trp-P-2 was inhibited by cysteine. Fig. 4 shows the effect of cysteine on the decrease in the amount of Trp-P-2. Cysteine inhibited the metabolism of Trp-P-2 as determined by the substrate disappearance. However, the inhibition of the accumulation of *N*-OH-Trp-P-2 by cysteine was greater than the inhibition of substrate disappearance. These results apparently indicate that cysteine suppressed the accumulation of *N*-OH-Trp-P-2 rather than inhibiting the oxidation of Trp-P-2.

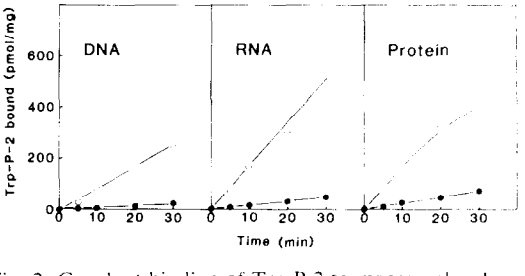

Fig. 2. Covalent binding of Trp-P-2 to macromolecules of untreated (●) or MC-pretreated (○) rat hepatocytes. The values indicate pmoles Trp-P-2 bound/mg for each macromolecule.

Table 1. Effects of 7,8-benzoflavone (BF) and diethylmaleate (DEM) on the binding of Trp-P-2 to DNA of MC-pretreated rat hepatocytes

	Concn of Trp-P-2 (μM)	Trp-P-2 bound (pmoles/mg DNA)	% of control
Expt. 1	30		
Control		380 ± 53	100
BF (0.5 mM)		35 ± 14	9.2
DEM (1.5 mM)		589 ± 93	155
Expt. 2	15		
Control		290 ± 9	100
BF (0.5 mM)		10 ± 2	3.4
DEM (1.5 mM)		437 ± 9	151

Incubation time was 30 min. The values are the means ± S.E.M. for triplicate determinations.

DISCUSSION

The metabolism of Trp-P-2 was much lower in hepatocytes from untreated rats than in hepatocytes from MC-pretreated rats. Consequently, the covalent binding of Trp-P-2 to DNA, RNA and protein in hepatocytes from untreated rats was much lower than that in hepatocytes from MC-pretreated rats. These results were in agreement with the data obtained from Ames tests showing that the mutagenic activation of Trp-P-2 by microsomes from untreated rats was much lower than that by microsomes from MC- or polychlorinated biphenyl treated rats [24]. BF inhibited the covalent binding to DNA and the metabolism of Trp-P-2. Since BF has been reported to preferentially inhibit the P-448 type of cytochrome P-450 [25, 26], Trp-P-2 seems to be oxidized by the P-448 type of cytochrome P-450 which is induced by MC. It has been reported that BF also inhibits the binding of benzo[*a*]pyrene to DNA of hepatocytes from MC-pretreated rats [10]. BF (0.5 mM) inhibited about 50% of the binding of benzo[*a*]pyrene [10], whereas the same concn of BF inhibited more than 90% of the binding of Trp-P-2. These results suggest that the substrate specificity of Trp-P-2 to BF-sensitive cytochrome P-450s is higher than that of benzo[*a*]pyrene.

The active intermediate, which is formed by microsomal [24] or nuclear [27] cytochrome P-450, has been identified as *N*-OH-Trp-P-2. It has been demonstrated that *N*-OH-Trp-P-2 can bind nonenzymatically to DNA [9, 28]. It has also been suggested that *N*-OH-Trp-P-2 is further activated and binds to DNA. For example, aminoacyl-tRNA synthetase could catalyze the binding of *N*-OH-Trp-P-2 to DNA [29]. Acetylation of *N*-OH-Trp-P-2 may also facilitate the binding of *N*-OH-Trp-P-2 to DNA [30]. Thus, the mechanisms of the binding of Trp-P-2 to DNA are complicated, but the essential intermediate for the covalent binding is *N*-OH-Trp-P-2. The addition of DEM, which is known to react with sulfhydryl compounds such as glutathione, enhanced by about 50% the covalent binding of Trp-P-2 to DNA. DEM seemed to inhibit the detoxification of Trp-P-2 by sulfhydryl compounds, and to consequently enhance the binding of Trp-P-2 to DNA. This explanation was supported by the discovery that cysteine inhibited the binding of Trp-P-2 to DNA. Cysteine itself or glutathione, which is readily synthesized from cysteine in hepatocytes [31, 32], seemed to react with metabolites of Trp-P-2 and inhibit the binding of Trp-P-2 to DNA. This effect of cysteine seemed due to stimulation of detoxification of *N*-OH-Trp-P-2 rather than due to inhibition of the oxidation of Trp-P-2 by cytochrome P-450. The substrate (Trp-P-2) disappearance appeared to be slightly inhibited by cysteine, but the inhibitory effect of cysteine on the accumulation of *N*-OH-Trp-P-2 was greater than the retardation of the substrate disappearance. It is not clear which mechanisms are involved in the detoxificative effects of cysteine. However, it is conceivable that cysteine maintained a high concn of intracellular glutathione and enhanced the conjugation of glutathione with *N*-OH-Trp-P-2 or other metabolites, such as the nitroso form of Trp-P-2. Cysteine itself may also affect the fate of *N*-OH-Trp-P-2.

Further, it is not clear why cysteine caused a small but significant retardation of the disappearance of Trp-P-2. It may be possible that cysteine or gluta-

Table 2. Effect of cysteine on the binding of Trp-P-2 to DNA of MC-pretreated rat hepatocytes

Concn of cysteine (mM)	Trp-P-2 bound (pmoles/mg macromolecules)		
	DNA	RNA	Protein
0	249 ± 10 (100)	716 ± 37 (100)	405 ± 14 (100)
0.3	205 ± 8 (82)	693 ± 46 (97)	341 ± 21 (84)
1	174 ± 8 (70)	590 ± 29 (82)	366 ± 5 (90)
3	124 ± 2 (50)	236 ± 10 (33)	281 ± 15 (69)

Incubation time was 30 min. The numbers in parentheses indicate % of control. The values are the means ± S.E.M. for triplicate determinations.

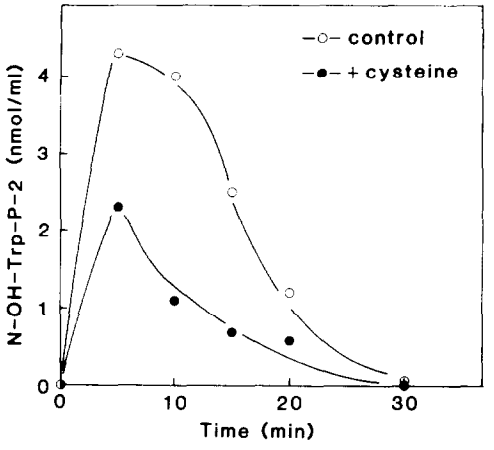

Fig. 3. Effect of cysteine on the amount of *N*-OH-Trp-P-2 formed by MC-pretreated rat hepatocytes. Concn of cysteine was 3 mM.

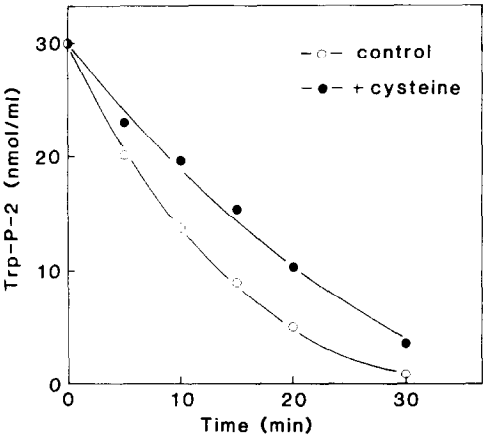

Fig. 4. Effect of cysteine on the metabolism of Trp-P-2 by MC-pretreated rat hepatocytes. Concn of cysteine was 3 mM.

thione reduced an oxidized form(s) of Trp-P-2 back into Trp-P-2.

It has been reported that cysteine enhances the mutagenic activity of Trp-P-2 in the Ames test in the presence of hepatic post-mitrochondrial fractions [33]. In the present study, cysteine reduced the accumulation of the active metabolite, *N*-OH-Trp-P-2, and the binding of Trp-P-2 to DNA. The reason for this apparent discrepancy was not clear. The metabolic activation by *in vitro* systems may be different from the conditions that exist within intact cells.

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