Vol. 104, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1982 Pages 193-199

IMMUNOLOGICAL ANALYSIS OF THE ROLES OF TWO MAJOR TYPES OF CYTOCHROME P-450 IN MUTAGENESIS OF COMPOUNDS ISOLATED FROM PYROLYSATES

Junko Watanabe, Kaname Kawajiri, Hiromichi Yonekawa, Minako Nagao* and Yusaku Tagashira

Department of Biochemistry, Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama 362, and *Biochemistry Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104, Japan.

Received November 20, 1981

The roles of two major types of cytochrome P-450 in the metabolic activation of ten compounds isolated from pyrolysates were examined in Ames' test system with specific antibodies against respective major types of cytochrome P-450 of rat liver microsomes induced by phenobarbital(PB) and 3-methylcholanthrene(MC), which are called PB-P-450 and MC-P-448. The mutagenic activation of all ten compounds derived from pyrolysates of tryptophan, glutamic acid, lysine, broiled sun-dried sardines and soy bean globulin by the S-9 fraction prepared from rat liver treated with polychlorinated biphenyls was inhibited more than 70% by antibody to MC-P-448. However, none were inhibited appreciably by antibody to PB-P-450, except lysine pyrolysate, which was inhibited about 20%. Furthermore, the mutagenicity of each compound tested was completely inhibited by antibody to NADPH-cytochrome P-450 reductase. It is concluded that the pyrolysis products examined in this study are activated to form mutagenic intermediates selectively by MC-P-448 in microsomes.

INTRODUCTION

Pyrolysates of proteins showed mutagenicity (1,2) and ten heterocyclic aromatic amines and a heterocyclic aromatic hydrocarbon were isolated from pyrolysates as potent mutagens. Among these, Trp-P-1 and Trp-P-2 were found to be hepatocarcinogenic (3,4,5), and much attention has paid to them as possible cause of human cancer. As is the case for many other mutagenic compounds, metabolic activation is needed for these pyrolysates to exert their mutagenic effects on the organism (6). The involvement of microsomal cytochrome P-450-dependent monooxygenase in the activation of Trp-P-1 and Trp-P-2 to mutagenic

Abbreviations: PB, phenobarbital; MC, 3-methylcholanthrene; PCB, polychlorinated biphenyls; Trp-P-1, 3-amino-1,4-dimethyl-5H-prido[4,3-b]indole; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole; Glu-P-1, 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole; Glu-P-2, 2-amino-dipyrido[1,2-a:3',2'-d]-imidazole; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline; AαC, 2-amino-9H-pyrido[2,3-b]indole; MeAαC, 2-amino-3-methyl-9H-pyrido[2,3-b]indole; 3-acetyl-Trp-P-1, 3-acetamide-1-methyl-5H-pyrido[4,3-b]indole; Lys-P-1, 3,4-cyclopentenopyrido[3,2-a]calbazole.

0006-291X/82/010193-07$01.00/0

intermediates has recently been demonstrated using a reconstituted monooxygenase system (7).

However, there are various forms of cytochrome P-450 in the microsomal membrane and the contents of each of these forms are varied by the administration of chemical compounds (8). Thus, it is important to determine what type of cytochrome P-450 is responsible for the mutagenesis of pyrolysis products in the whole microsomal membrane.

We previously showed the roles of two major types of microsomal cytochrome P-450 in the mutagenesis of various carcinogens by use of specific antibodies against PB-P-450 and MC-P-448, and found that the contributions of the two types of cytochrome P-450 to the metabolic activation differ for various carcinogens (9).

In this study, we investigated the contributions of the two types of cytochrome P-450 to mutagenesis by ten chemicals from pyrolysates of amino acids, protein, and proteinous foods using specific antibodies to these two cytochrome P-450 species, and found that all the mutagens are activated selectively by MC-P-448 in microsomes.

MATERIALS AND METHODS

Preparation of S-9 fraction and microsomes from rat liver: Male Sprague-Dawley rats weighing 200 to 300g were used. PCB (Kanechlor KC 500) was injected intraperitoneally into rats once daily for three successive days at 100mg/kg body weight. The S-9 fraction and microsomes from PCB-treated rat liver were prepared as described previously (9).

Analytical procedures: The content of total cytochrome P-450 in the microsomes was found to be 166.0μg/mg protein by the CO-differential spectrophotometric method (10). The contents of PB-P-450 and MC-P-448 were analyzed by the immunoprecipitation method (11), and found to be 75.0μg/mg and 60.0 μg/mg, respectively. Protein was determined by the method of Lowry et al. (12).

Preparation of specific antibodies against microsomal electron transport components: PB-P-450 and MC-P-448 were purified from the liver microsomes of rats pretreated with phenobarbital and 3-methylcholanthrene , respectively, as described by Harada and Omura (11). The specific contents of the two purified preparations were about 17.0nmoles/mg of protein. NADPH-cytochrome P-450 reductase was purified as described by Omura and Takesue (13). Preparation and purification of antibodies against three microsomal electron transport components were carried out as described previously (14). Anti-PB-P-450 and anti-MC-P-448 immunoglobulin inhibited the benzphetamine-N-demethylase and arylhydrocarbon hydroxylase activities, respectively, of microsomes from PCB-treated rat liver (9). The results of an Ouchterlony immunodiffusion test are shown in Figure 1.

Mutagenesis assay in the presence of antibody: Salmonella typhimurium strain TA98 was used. Mutagenesis assay in the presence of antibody was carried out as described previously (9). Before the addition of mutagens, S-9

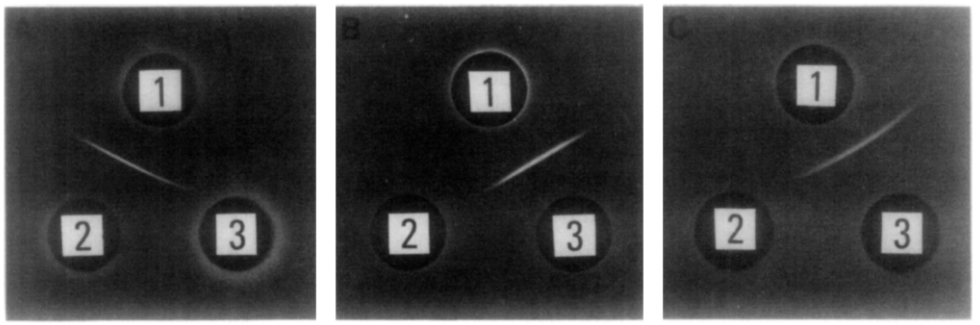


Figure 1. Ouchterlony immunodiffusion test of the antibodies. The experimental conditions were as described previously (9), except that sodium cholate was omitted from the gel plate with antibody to NADPH-cytochrome P-450 reductase. (A). Well 1 contained antibody to PB-P-450. Well 2 and 3 contained partially purified PB-P-450 and MC-P-448, respectively. (B). Well 1 contained antibody to MC-P-448. Wells 2 and 3 were the same as for (A). (C). Well 1 contained antibody to NADPH-cytochrome P-450 reductase. Wells 2 and 3 contained buffer and partially purified NADPH-cytochrome P-450 reductase, respectively.

fraction was preincubated with antibodies at 25°C for 20 min, and then mutagenicity was assayed by the preincubation method (15). The S-9 protein concentration used for each pyrolysis product was on the linear portion of the curve for inducing mutations. Mutagenesis assay was performed in duplicate. Experimental conditions for mutagenic assay of pyrolysis products in the presence of antibodies are showed in Table 1.

Chemicals: PCB (Kanechlor KC 500) was from Oriental Yeast Co, Tokyo. The 10 compounds tested for mutagenicity were kindly donated by Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo. Trp-P-1 and Trp-P-2, which were originally isolated from a tryptophan pyrolysate, were used in their acetate form. AαC and MeAαC, which were originally isolated as mutagens from soy bean globulin pyrolysate, were also used as their acetates. Glu-P-1 and Glu-P-2, which were isolated from a glutamic acid pyrolysate, were used as hydrochlorides. Lys-P-1, which was originally isolated from a lysine pyrolysate, IQ and MeIQ,isolated from broiled sun-dried sardines, and 3-acetyl-Trp-P-1 were used as their free forms. Other chemicals were standard products of reagent grade.

RESULTS

Inhibition of mutagenic activity by antibody to NADPH-cytochrome P-450 reductase. We studied the effect of antibody to NADPH-cytochrome P-450 reductase on the mutagenic activities of the 10 pyrolysis products, since this antibody inhibits electron flow between NADPH-cytochrome P-450 reductase and all forms of cytochrome P-450. Figure 2 shows the effect of the antibody on the mutagenic activities of Trp-P-1 and Trp-P-2. The mutagenicities of Trp-P-1 and Trp-P-2 were both inhibited 92% by a concentration of 24.5mg of antibody per nmole P-450. Similar inhibitory effects of the antibody were observed on the mutagenic activities of other pyrolysis products (data not shown). It was

Table 1

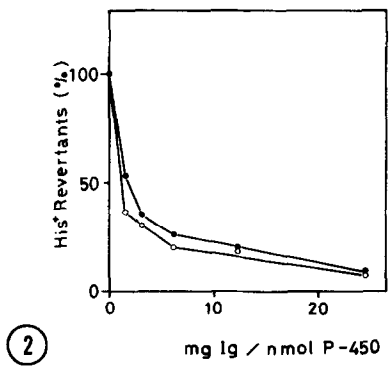
Experimental conditions for assay of mutagenesis of pyrolysis products in the presence of antibodies

Pyrolysis products	Trp-P-1	Trp-P-2	Glu-P-1	Glu-P-2	IQ	MeIQ	AαC	MeAαC	3-Acetyl-Trp-P-1	Lys-P-1
Dose (μg/plate)	0.05	0.03	0.5	3.5	0.02	0.005	2.5	6.0	5.0	8.0
Total P-450 (nmoles/plate)	0.19	0.19	0.31	0.31	0.30	0.30	0.52	0.52	0.30	0.50
No. of $His^{+(*)}$ revertants	2638	3882	17550	1736	3673	3257	1993	1326	1654	674

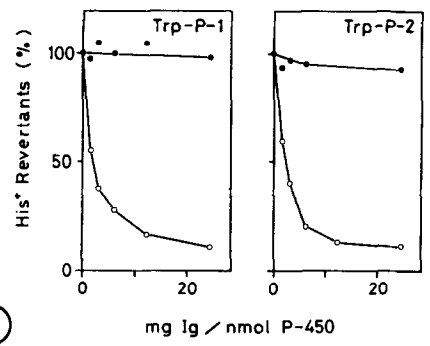
(*), in the presence of control Ig.

concluded that all the pyrolysis products used in this study were metabolized to mutagenically active forms by the microsomal electron transport system.

Effects of antibodies to PB-P-450 and MC-P-448 on the mutagenic activation. We examined the contribution of PB-P-450 and MC-P-448 to mutagenic activation of the 10 pyrolysis products using specific antibodies. Figure 3 shows the effects of the antibodies on the mutagenic activations of Trp-P-1 and Trp-P-2. The mutagenic activities of both compounds were inhibited 89% by a concentration of 24.5mg of antibody to MC-P-448 per nmoles P-450, whereas antibody to PB-P-450 hardly inhibited the mutagenic activation. In the same



(2)



(3)

Figure 2. Effect of antibody to NADPH-cytochrome P-450 reductase on the mutagenic activities of Trp-P-1 and Trp-P-2. Trp-P-1 (0.05μg) and Trp-P-2 (0.03μg) were incubated with 0.19nmoles of cytochrome P-450 in the S-9 fraction of PCB-treated rat liver per plate in the presence of the antibody. The number of His^+ revertants of Trp-P-1 and Trp-P-2 in the presence of control immunoglobulin were 2368 and 3882, respectively. ○, Trp-P-1; ●, Trp-P-2.

Figure 3. Effects of antibodies to PB-P-450 and MC-P-448 on the mutagenic activities of Trp-P-1 and Trp-P-2. Experimental conditions were as described in the legend to Figure 2. The His^+ revertants in the presence of antibody to PB-P-450 (●) and MC-P-448 (○) are shown in the figures as percentages of the His^+ revertants assayed with control immunoglobulin.

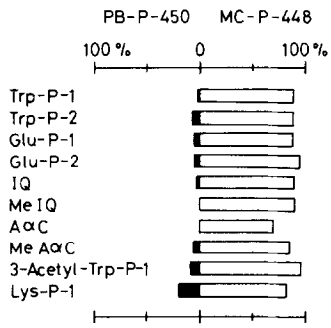


Figure 4. Dependency on metabolic activation of 10 pyrolysis products. This figure summarizes the effects of antibodies on the mutagenicities of the 10 pyrolysis products. The percentage inhibitions of mutagenicity in the presence of excess antibody to PB-P-450 (■) and MC-P-448 (□) are indicated. These values indicate the dependency of the mutagenicities on the corresponding molecular species of cytochrome P-450.

way, we examined the roles of the two types of cytochrome P-450 in mutagenesis by the other pyrolysis products. The inhibitions of various pyrolysis products by the antibodies against the two types of cytochrome P-450 are summarized in Figure 4. Judging from this figure, we conclude that the pyrolysis products used in this study are activated selectively by MC-P-448 in the microsomes of rat liver.

DISCUSSION

In this work, using specific antibodies against microsomal electron transport components, we studied the first step of metabolic activation of 10 compounds obtained from pyrolysates by Ames' test system. We demonstrated directly the involvement of the microsomal electron transport system in the activation of these pyrolysis products by showing that the mutagenic activities of Trp-P-1 and Trp-P-2 are completely inhibited by antibody to NADPH-cytochrome P-450 reductase (Figure 2). NADPH-cytochrome P-450 reductase is present as a single enzyme and mediates in electron transfer from NADPH to all forms of cytochrome P-450 on microsomal membranes (8). Thus, the rate of inhibition of mutagenic activity of the carcinogen by antibody to NADPH-cytochrome P-450 reductase should indicate the total activities mediated by various forms of cytochrome P-450, even if different molecular species of cytochrome P-450 participate in the mutagenic activity of each carcinogen.

Although similar concentrations of PB-P-450 and MC-P-448 are present in microsomes from PCB-treated rat liver, antibody to MC-P-448 inhibited the mutagenic activities of all the compounds tested more than 70%, whereas antibody to PB-P-450 was scarcely inhibitory. Thus, we conclude that all the pyrolysis products tested were selectively activated to mutagenic forms by MC-P-448 in liver microsomes. These results obtained with specific antibodies are consistent with the results of Ishii et al. (7) and Nebert et al. (16) obtained with a reconstituted system of microsomal monooxygenase and by the genetic approach, respectively. Furthermore, since mutagenic activation was inhibited about 90% by excess antibodies to NADPH-cytochrome P-450 reductase and to MC-P-448, the other minor components of cytochrome P-450 present in microsomes of PCB-treated rat liver do not participate in the metabolic activation of pyrolysis products.

ACKNOWLEDGMENT

We thank Dr. T. Sugimura, National Cancer Center Research Institute, Tokyo, for supplying the compounds isolated from pyrolysates. This work was supported by a grant from the Japanese Ministry of Education, Science and Culture.

REFERENCES

1. Sugimura, T., Nagao, M., Kawachi, T., Honda, M., Yahagi, T., Seino, Y., Sato, S., and Matsukura, N. (1977) Origin of Human Cancer, pp. 1561-1577, Cold Spring Harbor, New York.
2. Nagao, M., Yahagi,T., Kawachi,T., Seino, Y., Honda, M., Matsukura, N., Sugimura, T., Wakabayashi, K., Tsuji, K., and Kosuge,T. (1977) Progress in Genetic Toxicology, pp. 259-264, Elsevier/North-Holland Biomedical Press.
3. Ishikawa, T., Takayama, S., Kitagawa, T., Kawachi, T., Kinebuchi, M., Matsukura, N., Uchida, E., and Sugimura, T. (1979) Naturally Occurring Carcinogens-Mutagens and Modulators of Carcinogenesis, pp. 159-167, Japan Sci. Soc. Press, Tokyo/Univ. Park Press, Baltimore.
4. Matsukura, N., Kawachi, T., Morino, K., Ohgaki, H., and Sugimura, T. (1981) Science, 213, 346-347.
5. Hosaka, S., Matsushima, T., Hirono, I., and Sugimura, T. (1981) Cancer Letters, 13, 23-28.
6. Sugimura, T., Kawachi, T., Nagao, M., Yamada, M., Takayama, S., Matsukura, N., and Wakabayashi, K. (1980) Biochemical and Medical Aspects of Tryptophan Metabolism, pp. 297-310, Elsevier/North-Holland Biomedical Press.
7. Ishii, K., Ando, M., Kamataki, T., Kato, R., and Nagao, M. (1980) Cancer Letters, 9, 271-276.
8. Omura, T. (1978) Cytochrome P-450, pp. 138-163, Kodansha, Tokyo/Academic Press, New York.
9. Kawajiri, K., Yonekawa, H., Harada, N., Noshiro, M., Omura, T., and Tagashira, Y. (1980) Cancer Res., 40, 1652-1657.
10. Omura, T., and Sato, R. (1964) J. Biol. Chem., 239, 2379-2385.

11. Harada, N., and Omura, T. (1981) J. Biochem., 89,237-248.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem., 193, 265-275.
13. Omura, T., and Takesue, S. (1970) J. Biochem., 67, 249-257.
14. Noshiro, M., and Omura, T. (1978) J. Biochem., 83, 61-77.
15. Sugimura, T., and Nagao, M. (1980) Chemical Mutagens, Vol. 6, pp. 41-60. Plenum Publishing Corporation.
16. Nebert, D.W., Bigelow, S.W., Okey, A.B., Yahagi, T., Mori, Y., Nagao, M., and Sugimura, T. (1979) Proc. Natl. Acad. Sci. USA., 76, 5923-5933.