Contributions of Two Inducible Forms of Cytochrome P-450 in Rat Liver Microsomes to the Metabolic Activation of Various Chemical Carcinogens

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ABSTRACT

Using the liver 9000 × g supernatant fraction of uninduced rats and monospecific antibodies against microsomal reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase, and two inducible forms of cytochrome P-450, PB-P-450 (major cytochrome P-450 component of liver microsomes of phenobarbital-treated rats) and MC-P-448 (major cytochrome P-450 component of liver microsomes of 3-methylcholanthrene-treated rats), in the Ames test system, the contributions of these 2 forms of cytochrome P-450 to the mutagenicities of 3-amino-1-methyl-5H-pyrido[4,3-b]indole, 2-acetylaminofluorene, and aflatoxin B1 were studied. The major cytochrome P-450 component of the liver microsomes of 3-methylcholanthrene-treated rats, in the 9000 × g supernatant fraction of uninduced rats, was completely inhibited by antibody to MC-P-448 (anti-MC-P-448 immunoglobulin) and also inhibited 5, 30, and 60%, respectively, by antibody to PB-P-450 (anti-PB-P-450 immunoglobulin). These results indicate the importance of these two forms of cytochrome P-450 in the activation of the carcinogens by the liver 9000 × g supernatant fraction of uninduced rats.

We also examined the correlation between the induction of the two forms of cytochrome P-450 and the change of mutagenic activities at various time points after a single dose of various carcinogens to rats. Benzo(a)pyrene and 3-methylcholanthrene induced only MC-P-448 about 15 and 20 times, respectively, as much as that in untreated rats at the maximal levels of induction. The 9000 × g supernatant-mediated mutagenicities of benzo(a)pyrene and 3-methylcholanthrene varied in parallel with the content of each of the various forms of cytochrome P-450 in microsomes, and the induced mutagenicity of benzo(a)pyrene was completely inhibited by anti-MC-P-448 immunoglobulin. O-Aminazo- tolouene induced both MC-P-448 and PB-P-450 about 10 and 5 times as much, respectively, while the induced mutagenicity of o-aminazo- tolouene was inhibited 90 and 10% by the antibodies to MC-P-448 and PB-P-450, respectively. Both MC-P-448 and mutagenic activity of 2-acetylaminofluorene were induced about three times by a single dose of 2-acetylaminofluorene to rats. Administration of aflatoxin B1 showed neither induction of cytochrome P-450 nor an increase of mutagenic activity of aflatoxin B1. It is concluded that there is a good correlation between the increase of mutagenic activity and the content of the two cytochrome P-450 species in microsomes by administration of various carcinogens to rats.

INTRODUCTION

Cytochrome P-450 is the terminal oxidase of the microsomal electron transport system responsible for the oxidative metabolism of steroids, fatty acids, drugs, and other lipophilic xeno- biotics (12). It has been demonstrated that the cytochrome is also involved in metabolic activation of chemical carcinogens (2). This broad substrate specificity has been accounted for in part by the existence of multiple forms of this cytochrome in liver microsomes (6, 12).

Since metabolic activation of chemical carcinogens is an obligatory step in the development of chemical carcinogenesis, it is important to determine what molecular species of cytochrome P-450 contribute to the activation by microsomes. Using the S-93 fraction of PCB-treated rats and specific antibodies to 2 inducible forms of cytochrome P-450, PB-P-450 and MC-P-448, we previously showed that many chemical carcinogens were metabolically activated by these 2 cytochrome P-450 species and that the contributions of these 2 cytochromes to mutagenic activation differ with various chemical carcinogens (3, 4, 15).

However, selective increases of inducer-specific forms of cytochrome P-450 by treating the animals with different chemical compounds, including some chemical carcinogens, have also been established in recent years (6, 12). Consequently, the content of each of the various forms of cytochrome P-450 in microsomes is quite variable according to the induced states of animals (1, 13, 14). Thus, it is reasonable to assume that the contribution of individual forms of cytochrome P-450 to the metabolic activation of carcinogens in liver cells is dependent not only on its catalytic activity but also on its content in the liver microsomes of carcinogen-treated animals.

In this study, we investigated the correlation between the contents of 2 individual forms of cytochrome P-450 and the change of the mutagenic activity of a carcinogen at various time points after a single dose of the carcinogen to rats. We found that there is a good correlation between the induction of an individual form of cytochrome P-450 and the increase of mutagenic activity by administration of chemical carcinogens to rats.

The abbreviations used are: S-9 fraction, 9,000 × g supernatant fraction; PCB, polychlorinated biphenyls; PB-P-450, major cytochrome P-450 component of liver microsomes of phenobarbital-treated rats; MC-P-448, major cytochrome P-450 component of liver microsomes of 3-methylcholanthrene-treated rats; P-448, major cytochrome P-450 component of liver microsomes of 3-methylcholanthrene-treated rats; OAT, o-aminoozotoluene; 2-AAF, 2-acetylaminofluorene; AFB1, aflatoxin B1; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole.

1 This work was supported in part by a Grant-in-Aid for Cancer Research from the Japanese Ministry of Education, Science, and Culture.

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Received May 25, 1982; accepted November 3, 1982.
MATERIALS AND METHODS

Preparation of S-9 Fraction and Microsomes from Rat Liver.

Sprague-Dawley rats weighing 150 to 200 g were used. Three rats were used at each time point of the induction experiments. B(a)P (25 mg/kg of body weight), 3-MC (25 mg/kg of body weight), and OAT (200 mg/kg of body weight) dissolved in corn oil were injected i.p. into rats. 2-AAF (100 mg/kg of body weight) and AFB1 (0.1 mg/kg of body weight) dissolved in a mixture of DMSO and corn oil were injected i.p. into rats. After the injection, microsomes and S-9 fraction were prepared as described previously (4) at various time points as described in the text. Uninduced microsomes and S-9 fraction were prepared from the livers of uninduced rats.

Preparation of Specific Antibodies against 3 Microsomal Electron Transport Components. NADPH-cytochrome P-450 reductase was purified from rat liver microsomes as described by Omura and Takesue (9). PB-P-450 and MC-P-448 were purified from the liver microsomes of rats pretreated with phenobarbital and 3-MC, respectively, as described by Harada and Omura (1). Preparation of antibodies against each of these 3 purified antigens was carried out as described by Noshiro and Omura (7). The specificity of the antibodies used were confirmed as reported previously (4, 15).

Analytical Procedures. Mutagenesis assay using Salmonella typhimurium strain TA 98 was carried out as described previously (4). The content of total cytochrome P-450 in microsomes was determined by the method of Omura and Sato (8). The contents of PB-P-450 and MC-P-448 in microsomes were determined by immunoprecipitation method using antibodies as described by Harada and Omura (1). The immunoreaction mixture contained 3.8 mg of anti-PB-P-450 immunoglobulin or 4.4 mg of anti-MC-P-448 immunoglobulin and corresponding antigens in 2 ml of 10 mM potassium phosphate buffer (pH 7.5) containing 0.9% NaCl, 0.25% sodium cholate, and 0.25% Emulgen 913. The assay mixture was incubated at 37 °C for 30 min and then at 4 °C for 2 days. The immunoprecipitates were collected by centrifugation at 3000 rpm for 10 min and washed twice with solubilizing solution. They were washed further with 10 mM potassium phosphate buffer (pH 7.5) containing 0.9% NaCl, and protein determinations of the precipitates were carried out by the method of Lowry et al. (5). The amounts of immunoprecipitates formed increased linearly to the antigens in a range between 5 and 30 µg. Microsomes (0.5 mg of protein per ml) were solubilized with 0.5% sodium cholate and 0.5% Emulgen 913 in 10 mM potassium phosphate buffer (pH 7.5) containing 0.9% NaCl and centrifuged at 100,000 × g for 60 min. The solubilized microsomes were assayed for PB-P-450 and MC-P-448 by immunoprecipitation as described above.

Reagents and Chemicals. B(a)P was obtained from Sigma Chemical Co., St. Louis, Mo. 3-MC and OAT were obtained from Wako Pure Chemical Industries, Osaka, Japan. 2-AAF was obtained from Nakarai Chemicals, Kyoto, Japan. AFB1 was obtained from Makor Chemicals, Jerusalem, Israel. Trp-P-2 was a generous gift of Dr. T. Sugimura (National Cancer Center Research Institute, Tokyo, Japan).

RESULTS

Effects of Antibodies to Microsomal NADPH-Cytochrome P-450 Reductase, PB-P-450, and MC-P-448 on the Mutagenic Activity of Carcinogens Mediated by Liver S-9 Fraction of Uninduced Rats. Studies were made on the effect of the antibodies to microsomal NADPH-cytochrome P-450 reductase, PB-P-450, and MC-P-448 on the mutagenicities of Trp-P-2, 2-AAF, and AFB1 (Chart 1), since these 3 carcinogens have high mutagenic activities in the S-9 fraction of uninduced rats. The mutagenicities of these 3 carcinogens were completely inhibited by the antibody to NADPH-cytochrome P-450 reductase. Although each of PB-P-450 and MC-P-448 comprises only about 5% of total cytochrome P-450 in the microsomes of uninduced rats, the mutagenic activities of Trp-P-2, 2-AAF, and AFB1 were inhibited 85, 85, and 40% by the antibody to MC-P-448 and also 5, 30, and 60% by the antibody to PB-P-450, respectively. The inhibition profiles were similar to those using the S-9 fraction of PCB-treated rats (4, 15), in which both of the 2 cytochrome P-450s represent about 40% of the total cytochrome P-450 in the microsomes. These results indicate that these 2 forms of cytochrome P-450 are mainly responsible for the mutagenic activities of carcinogens mediated by liver S-9 fraction of uninduced rats.

Correlation between the Induction of Cytochrome P-450 and the Changes of Mutagenic Activities of Various Carcinogens. In the experiments shown in Chart 2a, a single dose of B(a)P was given to rats at time zero, and the contents of total cytochrome P-450, PB-P-450, and MC-P-448 in microsomes were analyzed at various time points. The amount of total cytochrome P-450 increased about 2 times at 48 hr after the injection of B(a)P to rats. However, the increase of MC-P-448 was much more marked (15 times at 48 hr) than that of total cytochrome P-450. On the other hand, PB-P-450 did not increase at all with this treatment. Chart 2b shows the mutagenic activity of B(a)P in the S-9 fraction prepared from rat liver.
Induction of Cytochrome P-450 and Activation of Carcinogens

Chart 2. a, changes in the contents of PB-P-450 and MC-P-448 in liver microsomes (Ms) induced by B(a)P. B(a)P was injected i.p. into rats at time zero. Three rats were killed at each time point, and the contents of total cytochrome P-450 (●), PB-P-450 (○), and MC-P-448 (□) in the liver microsomes were assayed as described in "Materials and Methods." b, changes in the mutagenic activity of B(a)P in the liver S-9 fraction of B(a)P-treated rats. After a single dose of B(a)P to rats, S-9 fraction was prepared at various time points. A fixed amount of S-9 fraction, 2 mg of protein, was incubated with various amounts of B(a)P as indicated in the chart, and strain TA98 his r revertants were assayed at the following times after the injection of B(a)P into rats. S-9 fraction of uninduced rats; ○, 1 day; ●, 2 days; △, 3 days; □, 5 days. c, effects of antibodies on the mutagenicity of B(a)P in the liver S-9 fraction of B(a)P-treated rats. Experimental conditions were the same as described in the legend to Chart 1. S-9 fraction was prepared from B(a)P-treated rats 48 hr after the injection of B(a)P. B(a)P, 5 μg, was incubated with 2 mg of the S-9 fraction per plate in the presence of antibodies. There were 600 strain TA98 his r revertants in the presence of control immunoglobulin; ○, anti-PB-P-450 immunoglobulin; △, anti-MC-P-448 immunoglobulin.

Chart 3. a, changes in the contents of PB-P-450 and MC-P-448 in the liver microsomes (Ms) of 3-MC-treated rats. 3-MC was injected i.p. into rats at time zero, and the contents of total cytochrome P-450 (●), PB-P-450 (○), and MC-P-448 (□) in the liver microsomes were assayed as described in "Materials and Methods." b, changes in the mutagenic activity of 3-MC in the liver S-9 fraction of rats given injections of a single dose of 3-MC. After the injection of 3-MC into rats, S-9 fraction was prepared at various time points after the injection of 3-MC. △, S-9 fraction of uninduced rats; ○, 1 day; ●, 2 days; △, 3 days; □, 5 days. A fixed amount of S-9 fraction, 2 mg, was incubated with various amounts of 3-MC as described in the chart, and strain TA98 his r revertants were assayed.

at various time points after the single dose of B(a)P. The mutagenic activity of B(a)P at the maximal point in S-9 prepared at 24 and 48 hr after the injection increased, respectively, about 8 and 17 times more than that of untreated rats. The mutagenicity then was gradually decreased to the level in uninduced rats. Chart 2c shows the effect of antibodies against PB-P-450 and MC-P-448 on the mutagenic activity of B(a)P in the S-9 fraction at 48 hr after the injection. Anti-MC-P-448 immunoglobulin completely inhibited the mutagenicity, while anti-PB-P-450 immunoglobulin did not inhibit it at all. These results indicate that induced mutagenic activity of B(a)P was dependent on the content of MC-P-448 in microsomes.

Chart 3 shows the induction of cytochrome P-450 and the change of mutagenic activity of 3-MC by the administration of 3-MC to rats. The results were very similar to those of B(a)P (Chart 2).

Chart 4a shows the contents of total P-450, PB-P-450, and MC-P-448 in liver microsomes prepared from OAT-treated rats. Total cytochrome P-450 increased only by about 30% at 24 hr after a single injection of OAT. However, MC-P-448 and PB-P-450 increased about 10 and 5 times respectively, at 24 hr. The mutagenic activity of OAT also increased markedly in parallel with these 2 forms of cytochrome P-450 in microsomes at 24 and 48 hr after the injection (Chart 4b). We determined what type of cytochrome P-450 contributed to the activation of OAT in the S-9 fraction of OAT-induced rats. Anti-MC-P-448 immunoglobulin and anti-PB-P-450 immunoglobulin inhibited the mutagenic activity of OAT by 90 and 10%, respectively (Chart 4c). These results showed the major contribution of MC-P-448 to the metabolic activation of OAT in the livers of OAT-induced rats.

When a single dose of 2-AAF was given to rats at time zero, the content of total cytochrome P-450 did not change between 24 and 120 hr after the injection (Chart 5a). The same was true for the induction of PB-P-450. However, the content of MC-P-448 increased in microsomes about 3 times at 24 hr. Chart 5b shows the mutagenic activity of 2-AAF in the S-9 fraction prepared from 2-AAF-induced rats. The mutagenic activity of 2-AAF at 24 hr was about 3 times greater than that of untreated rats.

Chart 6 shows the induction of cytochrome P-450 and the change of mutagenic activity of AFB1 by the injection of AFB1. AFB1 showed neither induction of cytochrome P-450 nor an increase in the mutagenic activity of AFB1 in our experimental system.

DISCUSSION

The first point to be emphasized is the large contribution of PB-P-450 and MC-P-448 to mutagenesis by various chemical carcinogens in the S-9 fraction of uninduced rats. As determined by an immunoprecipitation method, each of these 2 forms of cytochrome P-450 occupied only about 5% of the total cytochrome P-450 in the microsomes of the S-9 fraction, which coincided with the results of Thomas et al. (13, 14) and Harada et al. (1). In spite of the low contents of these 2 forms in the total cytochrome P-450 in the liver microsomes of uninduced rats, mutagenesis of Trp-P-2, 2-AAF, and AFB1, catalyzed by the S-9 fraction was almost completely inhibited by
the antibodies to PB-P-450 and MC-P-448 (Chart 1). Since the sum of the inhibition percentages by these antibodies was almost equal to the inhibition by the antibody to NADPH-cytochrome P-450 reductase, PB-P-450 and MC-P-448 are mainly responsible for the metabolic activation of chemical carcinogens in the livers of uninduced rats. These results suggest that the low mutagenic activity of carcinogens in the S-9 fraction of uninduced rats is due to low concentrations of PB-P-450 and MC-P-448 in the liver microsomes of uninduced rats.

Our immunochemical determinations of the contributions of PB-P-450 and MC-P-448 to the metabolic activation of carcinogens depended upon the specificity of the antibodies used. Recent studies by Levin et al. (10, 11) on the multiple forms of cytochrome P-450 in rat liver microsomes have confirmed immunological cross-reactivity among some of them. P-450b, which is the major form of cytochrome P-450 in the liver microsomes of phenobarbital-treated rats, was immunologically identical with a minor form of cytochrome P-450, P-450e. The major form of the 3-MC-inducible cytochrome P-450, P-450c, showed partial immuno-cross-reactivity with a minor form, P-450d. In view of their observations, we cannot exclude the possibility of the reaction of our antibody preparations, anti-PB-P-450 and anti-MC-P-448 immunoglobulins, with some minor forms of cytochrome P-450 other than PB-P-450 and MC-P-448, respectively, although the specificity of the antibodies to corresponding antigens was confirmed by several immunochemical methods (4). However, PB-P-450 and MC-P-448 are the predominant forms of cytochrome P-450 in the liver microsomes of phenobarbital- and 3-MC-treated rats, respectively (1, 13, 14). It seems reasonable to assume that the inhibition of metabolic activation of carcinogens by anti-PB-P-450 and anti-MC-P-448 immunoglobulins was mainly caused by the reaction of the antibodies with corresponding major forms of cytochrome P-450 in the microsomes.

If PB-P-450 and MC-P-448 play a central role in the initiation of the mutagenicity of OAT in the S-9 fraction of OAT-treated rats. Experimental conditions were the same as described in the legend to Chart 1. S-9 fraction was prepared from OAT-treated rats at 24 hr after the injection of OAT. OAT, 12.5 μg, was incubated with 2 mg of the S-9 fraction per plate in the presence of antibodies. There were 250 strain TA 98 revertants in the presence of control immunoglobulin (g). •, anti-PB-P-450 immunoglobulin; ○, anti-MC-P-448 immunoglobulin.

Chart 4. a, changes in the contents of PB-P-450 and MC-P-448 in the liver microsomes (Ms) of OAT-treated rats. OAT was injected i.p. into rats at time zero, and the contents of total cytochrome P-450 (■), PB-P-450 (●), and MC-P-448 (○) were assayed at various time points as described in “Materials and Methods.” b, changes in the mutagenic activity of OAT in the liver S-9 fraction after a single dose of OAT to rats. After the injection of OAT into rats, S-9 fraction was prepared at various time points. A fixed amount of S-9 fraction, 2 mg, was incubated with various amounts of OAT as indicated in the chart, and the strain TA 98 his r revertants were assayed at the following times after the injection of OAT: ■, S-9 fraction of uninduced rats; ○, 1 day; ●, 2 days; ▲, 3 days; △, 5 days. c, effects of antibodies on the mutagenicity of OAT in the S-9 fraction of OAT-treated rats. Experimental conditions were the same as described in the legend to Chart 1. S-9 fraction was prepared from OAT-treated rats at 24 hr after the injection of OAT. OAT, 12.5 μg, was incubated with 2 mg of the S-9 fraction per plate in the presence of antibodies. There were 250 strain TA 98 his r revertants in the presence of control immunoglobulin (g). •, anti-PB-P-450 immunoglobulin; ○, anti-MC-P-448 immunoglobulin.

Chart 5. a, changes in the contents of PB-P-450 and MC-P-448 in the liver microsomes (Ms) of 2-AAF-treated rats. 2-AAF was injected i.p. into rats, and the contents of total cytochrome P-450 (■), PB-P-450 (●), and MC-P-448 (○) were assayed at various time points as described in “Materials and Methods.” b, changes in the mutagenic activity of 2-AAF in the liver S-9 fraction of rats given injections of a single dose of 2-AAF. After the injection of 2-AAF into rats, S-9 fraction was prepared at various time points. ■, S-9 fraction of uninduced rats at the following time points after the injection of 2-AAF. ○, 1 day; ●, 2 days; ▲, 3 days; △, 5 days. A fixed amount of S-9 fraction, 2 mg, was incubated with various amounts of 2-AAF as described in the chart, and the strain TA 98 his r revertants were assayed.

Chart 6. a, changes in the contents of PB-P-450 and MC-P-448 in the liver microsomes (Ms) of AFB1-treated rats. AFB1 was injected i.p. into rats, and the contents of total cytochrome P-450 (■), PB-P-450 (●), and MC-P-448 (○) in the liver microsomes were assayed as described in “Materials and Methods.” b, changes in the mutagenic activity of AFB1 in the liver S-9 fraction of rats given injections of a single dose of AFB1. After the injection of AFB1 into rats, S-9 fraction was prepared from the rats at various time points after the injection of AFB1. ■, S-9 fraction of uninduced rats; ○, 1 day; ●, 2 days; ▲, 3 days; △, 5 days. A fixed amount of S-9 fraction, 2 mg, was incubated with various amounts of AFB1, as indicated in the chart, and the strain TA 98 his r revertants were assayed.
of chemical carcinogenesis, their inducibility by the administration of a carcinogen must be an important factor in natural and experimental carcinogenesis. Our model experiments support this hypothesis; i.e., we found that there was a good correlation between the change of the contents of these 2 forms of cytochrome P-450 and that of mutagenic activity of a carcinogen at various time points after a dose of a carcinogen to rats (Charts 2 to 6). The inducibility of these 2 forms of cytochrome P-450 was different according to various chemical carcinogens. Judging from the results of immunoprecipitation of solubilized microsomes of various carcinogen-treated rats, we can divide carcinogens at least into 3 classes according to the inducibility of these 2 forms of cytochrome P-450: (a) those increasing selectively the level of MC-P-448 [B(a)P, 3-MC, 2-AAF]; (b) those increasing both MC-P-448 and PB-P-450 [OAT]; and (c) those having no effect on the levels of both MC-P-448 and PB-P-450 [AFB].

The results described above indicate that we must consider at least 2 factors in evaluating the contribution of a form of cytochrome P-450 to the metabolic activation of a specific chemical carcinogen in vivo: (a) specific catalytic activity of the cytochrome P-450 on the carcinogens; and (b) induction of the cytochrome P-450 by the carcinogens. If we assume that the specific activity of a form of cytochrome P-450 is constant irrespective of its induction by a carcinogen, then a quantitative estimation of its contribution to the mutagenicity of the carcinogen is possible. This assumption is actually supported by the findings that the antibodies to PB-P-450 and MC-P-448 inhibited the mutagenesis by Trp-P-2, 2-AAF, or AFB to the same extent in both PCB-treated rats (4, 15) and uninduced S-9 fraction (Chart 1), where the relative contents of PB-P-450 and MC-P-448 were almost the same but the PCB-treated microsomes contained nearly 10 times as much as each cytochrome P-450 species (13, 14).

As an example, we consider the case of OAT. The ratio of relative specific activity of PB-P-450 and MC-P-448 to OAT was 3:7 (4), while the ratio of their contents in liver microsomes of OAT-pretreated rats was 1:2 (Chart 4). From these ratios, we can calculate the contributions of PB-P-450 and MC-P-448 to the activation of OAT to be 3:14. This ratio coincides with the results using specific antibodies and the S-9 fraction of OAT-induced rats. Namely, the antibodies to PB-P-450 and MC-P-448 inhibited OAT-dependent mutagenesis by 15 and 85%, respectively, giving a ratio of 3:17 (Chart 4). Similar results were obtained using other chemical carcinogens. Thus, it is concluded that B(a)P, 3-MC, 2-AAF, and OAT are activated selectively by MC-P-448 in the liver microsomes of rats pre-treated with each of these carcinogens. On the other hand, AFB, seems to be preferentially activated by PB-P-450 in the liver microsomes of AFB-treated rats, because AFB did not induce cytochrome P-450 and it was predominantly activated by PB-P-450 in the microsomes of uninduced rats.

ACKNOWLEDGMENTS

We wish to thank Dr. T. Omura, Kyushu University, for his critical reading of our manuscript.

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