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Variations among Untreated Rabbits in Benzo(a)pyrene Metabolism and Its Modulation by 7,8-Benzoflavone

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SUMMARY

The metabolism of benzo(*a*)pyrene by rabbit liver microsomes can be stimulated or inhibited by 7,8-benzo(*a*)flavone (ANF) depending on the distribution of specific P-450 enzymes present within the microsomes. Treatment of rabbits with either 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or rifampicin leads to an increase of hepatic microsomal metabolism of benzo(*a*)pyrene. ANF stimulates the rate of benzo(*a*)pyrene metabolism catalyzed by microsomes isolated from rabbits treated with rifampicin by 3-fold. In contrast, ANF moderately inhibits the activity of microsomes from TCDD-treated rabbits. Variations in the benzo(*a*)pyrene hydroxylase activity of microsomes from untreated rabbits apparently reflect differences in the expression of P-450 1, a constitutive form of P-450. Thus, the benzo(*a*)pyrene hydroxylase activity of microsomes from untreated rabbits, which varies from 0.40 to 1.5 nmol/min/mg of protein, is directly correlated with the microsomal concentration of P-450 1. The metabolism of benzo(*a*)pyrene by microsomes containing high concentrations of P-450 1 is inhibited by a monoclonal antibody specific for this cytochrome to approximately the rate exhibited by microsomes with a low concentration of P-450 1. The benzo(*a*)pyrene activity stimulated by ANF in microsomes with a low concentration of P-450 1 is not inhibited by the monoclonal antibody. The activity of P-450 1 is inhibited by ANF at concentrations that stimulate other constitutive forms of P-450. Thus, ANF produces offsetting effects on benzo(*a*)pyrene metabolism in microsomes from untreated animals by stimulating the activity of at least one cytochrome and inhibiting P-450 1-mediated activity.

INTRODUCTION

The P-450 monooxygenases can catalyze the initial steps of both carcinogen activation and detoxification (1). This enzyme system consists of a large number of distinct forms of P-450 that are independently regulated by factors such as heredity, sex, tissue, and age (1–4). The P-450 enzymes are an attractive target for the modification of initiation pathways of carcinogenesis because their activities can be altered by the administration of a variety of exogenous compounds. The flavonoids represent a group of compounds which have been widely studied as inhibitors of carcinogenesis. They may inhibit this process by acting as inducers of specific forms of P-450 as well as by selectively inhibiting or stimulating P-450 catalyzed reactions (5–10).

The *in vitro* effects of flavonoids as modulators of benzo(*a*)pyrene metabolism have been demonstrated in several species by a number of investigators (5–10). Previous studies indicate that ANF[1] stimulates the metabolism of benzo(*a*)pyrene by rabbit and human liver microsomes (5). In contrast, ANF markedly inhibits the liver microsomal metabolism of benzo(*a*)pyrene when rats are pretreated with 3-methylcholanthrene (6). Additional studies demonstrate that, in human liver microsomes, several flavonoids stimulate metabolic activation of aflatoxin B_1 resulting in an increase in the formation of mutagenic products (7). Flavone can inhibit or stimulate benzo(*a*)pyrene metabolism catalyzed by reconstituted purified forms of cytochrome P-450 (11). For example, flavone appears to stimulate the metabolism of benzo(*a*)pyrene by rabbit P-450 3c and 4 and to inhibit metabolism by P-450 6 (11). Thus, different forms of P-450 exhibit divergent responses to the flavonoids. The purpose of this investigation was to determine how microsomal variations in the concentrations of individual P-450 isozymes could affect ANF modulated benzo(*a*)pyrene metabolism in untreated and treated rabbits.

EXPERIMENTAL PROCEDURES

Materials

7,8-Benzoflavone was obtained from Aldrich and [7,10-^{14}C] benzo(*a*)pyrene (58.5 mCi/mmol) was purchased from Amersham.

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[1] The abbreviations used are: ANF, α-naphthoflavone (7,8-benzoflavone); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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Nonimmune mouse IgG and bovine serum albumin were obtained from Sigma. Safety-Solve scintillant was purchased from Research Products International. The isolation of microsomes and pretreatment of animals are described elsewhere (12). New Zealand White rabbits were obtained from local breeders. Cytochrome P-450 forms 1 (13), 2 (14), 3b (15), 4 (14), and 6 (16) were isolated by published procedures. P-450 3c was purified according to Koop *et al.* (17) with slight modifications. Each preparation exhibited a single major band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The purified cytochromes were reconstituted with reductase in the presence of dilauroyl-L-α-lecithin (13–16). The monoclonal antibody to P-450 1, 1F11, was prepared as previously described (20).

Methods

Benzo(a)pyrene hydroxylase activity was determined by the modified radiometric method of Van Cantfort *et al.* (18). Rates were directly proportional to the concentration of microsomal protein over the range of 0.03–1.0 mg/ml. An incubation time of 15 min was selected for microsomal samples. In reconstitution experiments, the incubation times were reduced to 5–10 min in order to limit substrate depletion to less than 10%. The reaction media consisted of 30 μg of microsomal protein or, for reconstituted enzymes, 0.1 nmol of purified cytochrome, 0.12 nmol of reductase, and 30 μg of dilauroyl-L-α-lecithin, in a final volume of 1.0 ml of 50 mM potassium phosphate (pH 7.4) buffer containing 0.8 mg/ml bovine serum albumin. [^{14}C]Benzo(a)pyrene (0.5 mM) was dissolved in methanol, and 10-μl amounts were added to the incubation mixture to yield a final concentration of 5 μM. The observed rate of benzo(a)pyrene metabolism by microsomes diminished greatly when the concentration of substrate was lowered whereas only a moderate increase was observed when the concentration was increased above 5 μM. ANF was dissolved in methanol at a concentration of 1 mM and 10 μl was added to the reaction mixture just prior to addition of the substrate yielding a final concentration of 10 μM. An equal volume of methanol (10 μl) was added to the reaction mixture when ANF was excluded. Addition of the monoclonal antibody was made prior to that of the buffer, substrate, or effector. Nonimmune mouse IgG served as a control antibody. Microsomes or cytochrome and antibody were allowed to preincubate for 2–5 min at 4°.

The reactions were initiated by the addition of 20 μl of 50 mM NADPH (final concentration of 1.0 mM) and stopped by the addition of 1 ml of KOH (0.15 M in 85% dimethyl sulfoxide). The unmetabolized substrate was extracted in 5 ml of hexane. After centrifugation, the upper and interphases were removed and the aqueous phase was extracted again in a similar manner. An aliquot of the combined hexane phase was taken to determine total recovery and 0.2 ml of the aqueous phase was acidified by addition of an equal volume of 1 N HCl, diluted with 5 ml of scintillant and counted. When both phases were counted, recovery of substrate and metabolites was >90%. All incubations were performed in duplicate, and the mean of the two samples, corrected for background, was used to determine the enzymatic rate. The background levels (between 200 and 500 dpm) were determined for incubations where the enzyme was omitted or heat inactivated (the two procedures gave similar results). The enzymatic activity was expressed as nanomoles of total metabolites formed/min/mg of microsomal protein or per nmol of P-450.

Preparations of the antibodies. The antibodies utilized in this study were produced as described and purified by affinity chromatography on protein A-agarose (20). Antibodies used for inhibition studies and as labeled reagents were purified from serum-free media, HB101, harvested from confluent cultures of the respective hybridoma line. Antibodies used in the two-site immunoradiometric assay for coating plates were purified from ascites fluid by column chromatography following initial precipitation using ammonium sulfate (50% saturation). In order to label the antibody, 200 μg of the purified protein were reacted with 1 mCi of ^{125}I using the chloramine-T method. After extensive dialysis, greater than 90% of the ^{125}I was precipitated at a final concentration of 10% trichloroacetic acid, indicating extensive incorporation of the label.

Preparation of microsomes. Rabbit liver microsomes were prepared as previously described (12) and stored at −60°. Microsomal samples for immunoquantitation were diluted to 0.1 mg/ml in a solubilization buffer consisting of 50 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM Na_2EDTA, 0.2% sodium cholate, and 1.0% Nonidet P-40. After a 2-hr incubation at room temperature, the samples were diluted roughly 100-fold or greater in PBS containing 3% BSA. Purified cytochrome P-450 1 was prepared as described previously (13) and diluted in a similar manner. The rate of deoxycorticosterone formation from [^{14}C]progesterone was determined as described (13).

Two-site immunoradiometric assay. This assay is described in detail elsewhere (19). The wells of a 96-well microtest plate were coated with a monoclonal antibody by incubating in each well at 37° for 2 hr, 100 μl of 0.05 mg of IgG/ml in 0.1 M Na_2CO_3, pH 9.5. After washing with PBS, additional binding sites were blocked with 3% BSA/PBS. Samples of the purified cytochrome or solubilized microsomal protein were then incubated in the wells of the test plate overnight at room temperature. Microsomal samples (1–2 mg/ml) were solubilized in the presence of 1% Nonidet P-40 and 0.2% sodium cholate. The wells were then washed, and a subsequent incubation was performed with a solution containing a second monoclonal antibody diluted in 3% BSA/PBS for 3.5 hr at 37°. The second antibody recognizes an epitope distinct from the first and was labeled with ^{125}I. Unbound, labeled antibody was removed by aspiration, and the plate was washed and dried. The ^{125}I-antibody remaining in each well was counted and compared to a standard curve obtained for known quantities of P-450 1 (19).

RESULTS

Modulation of microsomal benzo(a)pyrene hydroxylase activity by ANF. The rate of metabolism of benzo(a)pyrene (5 μM) was examined for liver microsomes prepared from individual, untreated rabbits, and the basal activity was observed to vary over a 4-fold range, 0.35 to 1.5 nmol/min/mg of microsomal protein. In the presence of 10 μM ANF, the fractional increase in activity affected by ANF varied from 0 to 110%. Linear regression analysis revealed a negative correlation (r= −0.92) between the per cent increase in activity in the presence of ANF and the basal rate of benzo(a)pyrene metabolism (Fig. 1). The variations in basal activity suggested underlying differences among these microsomal preparations in the concentration of a P-450 enzyme(s) that catalyzes benzo(a)pyrene metabolism. Moreover, the inverse relationship to the degree of stimulation suggested that this P-450 might be inhibited by ANF at concentrations that stimulate the metabolism of benzo(a)pyrene by other P-450s. Thus, the stimulation of one cytochrome would be offset by the inhibition of another P-450 enzyme.

We have previously characterized differences in the concentration of a constitutive cytochrome, P-450 1, among microsomes prepared from untreated rabbits (19). Therefore, we examined whether these differences were related to the observed variation in benzo(a)pyrene metabolism. The rate of benzo(a)pyrene metabolism was found to be correlated directly (r = 0.93) to the microsomal concentration of P-450 1 estimated by immunoquantitation (19) (Fig. 2). The slope of the regression line yields a rate of 2.3 nmol of benzo(a)pyrene metabolized per min/nmol of P-450 1, and the intercept indicates a basal rate of 0.6 nmol/min/mg that can be attributed to other P-450 enzymes.

Effect of a monoclonal antibody to P-450 1 on microsomal benzo(a)pyrene metabolism. To confirm the role of P-450 1 in benzo(a)pyrene metabolism in microsomes from untreated animals, a monoclonal antibody that had

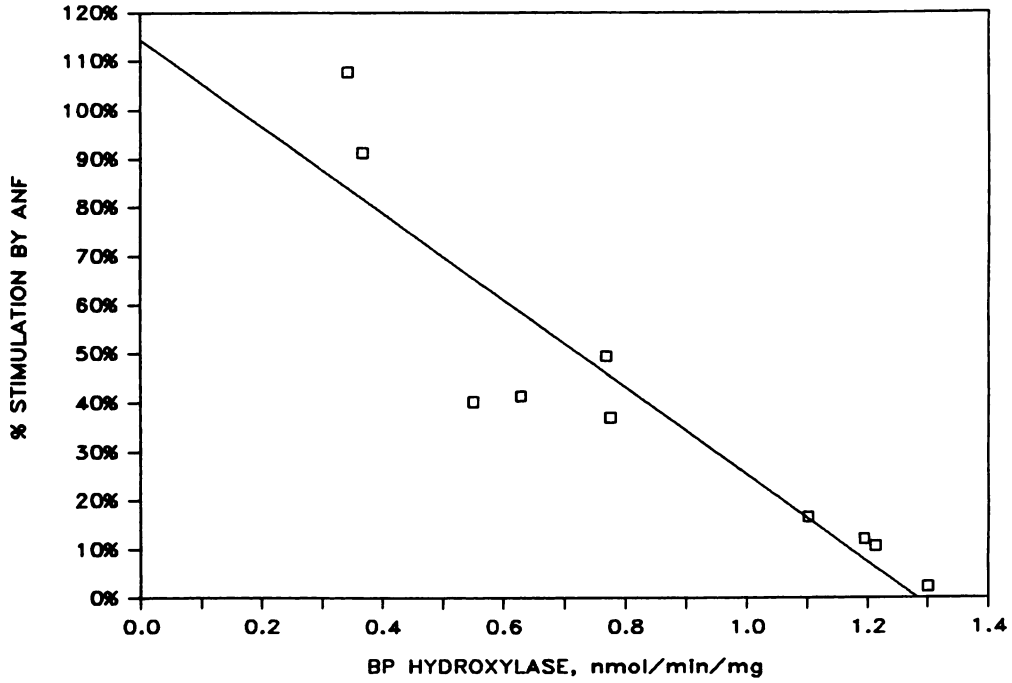

FIG. 1. *Correlation of microsomal benzo(a)pyrene hydroxylase activity and ANF stimulatable activity*

Linear regression analysis demonstrating a negative correlation between benzo(*a*)pyrene (*BP*) hydroxylase activity, (nanomoles/mg/min) and the additional activity observed when ANF is present, expressed as per cent stimulation, for liver microsomes from 10 individual untreated rabbits. A negative correlation coefficient of −0.92, a slope of −0.89, and an intercept of 1.28 nmol/mg/min were obtained.

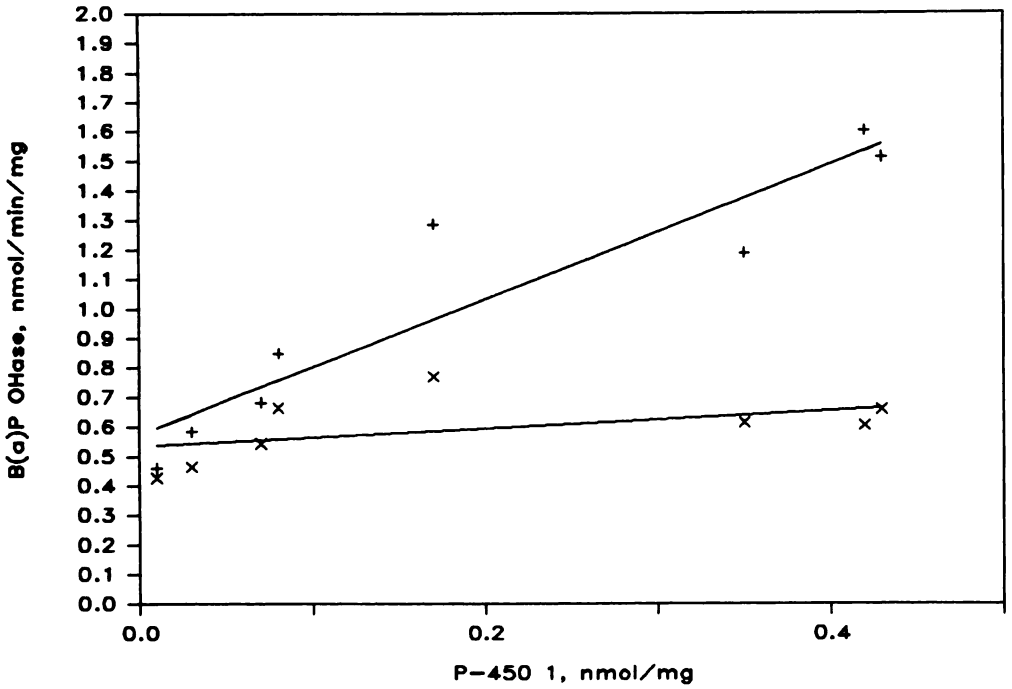

FIG. 2. *Correlation of microsomal cytochrome P-450 1 concentration and benzo(a)pyrene hydroxylase activity*

Linear regression analysis depicting a correlation between benzo(*a*)pyrene hydroxylase (*B(a)POHase*) activity (nanomoles/mg/min) and the concentration of P-450 1 (nanomoles/mg) in preparations of liver microsomes from eight individual untreated rabbits. Microsomes in the presence of 25 μg of nonimmune mouse IgG (represented by +) display a correlation coefficient of 0.93, a slope of 2.3 nmol/min/nmol P-450, and an intercept of 0.58 nmol/mg/min. When 25 μg of an inhibitory monoclonal antibody specific for P-450 1 (represented by ×) was incubated with the microsomes, the correlation was no longer evident (correlation coefficient < 0.5).

been prepared to P-450 1 was added to the incubation mixtures. This monoclonal antibody inhibits P-450 1-mediated 21-hydroxylation of progesterone and sequesters a single electrophoretic species from microsomes exhibiting a high rate of progesterone 21-hydroxylase activity (20). A titration curve utilizing various concentrations of the antibody indicated that the amount of antibody required to produce maximal inhibition in microsomes with a high concentration of P-450 1 was 5 μg (data not shown). Thus, the amount (25 μg) utilized in this study was in excess of that necessary to achieve maximal inhibition. Addition of the antibody to the reaction mixture demonstrated that the greatest extent of inhibition (46%) occurred in microsomes with a high concentration of P-450 1. Linear regression analysis of benzo(a)pyrene activity in the presence of the antibody demonstrated that the monoclonal antibody inhibits benzo(a)pyrene activity to the basal level of activity (Fig. 2), and the rate observed in the presence of the antibody was no longer correlated with the microsomal concentration of P-450 1 ($r < 0.5$).

Benzo(a)pyrene hydroxylase activity by purified cytochromes. When P-450 1 and several other purified P-450 isozymes were reconstituted with P-450 reductase, both purified P-450 1 and P-450 6 demonstrated high turnover numbers when compared to other purified cytochromes (Fig. 3). Cytochrome P-450 6, which is induced in rabbits treated with TCDD, displayed the highest rate with P-450 1 possessing 80% of the activity of P-450 6. When the P-450 1-specific antibody (25 μg) was added to the reconstituted cytochromes, P-450 1-mediated benzo(a)pyrene hydroxylase activity was depressed 95%, while the activity of the other cytochromes remained unchanged.

The addition of 10 μM ANF to the reconstituted purified cytochromes produced a 95% decrease in P-450 6-mediated benzo(a)pyrene activity while only a 50% inhibition was observed in the activity mediated by P-450 1. Further characterization indicated that the I_{50} for P-450 6 was 1000 times less than that for P-450 1. Only P-450 3c displayed an increase in benzo(a)pyrene metabolism when ANF was present.

The effect of ANF on microsomal metabolism of benzo(a)pyrene by microsomes from untreated, TCDD-, and rifampicin-treated animals. Since ANF produces differential effects on benzo(a)pyrene metabolism catalyzed by cytochromes P-450 3c and P-450 6, we examined the effect of ANF on benzo(a)pyrene metabolism catalyzed by microsomes containing elevated concentrations of either P-450 3c or P-450 6. Treatment of animals with TCDD elevates the liver microsomal concentrations of P-450 6 and P-450 4 (12), whereas rifampicin increases the concentration of P-450 3c (21).

Liver microsomes prepared from TCDD- or rifampicin-treated rabbits exhibit rates of benzo(a)pyrene metabolism that are similar to that catalyzed by microsomes containing high concentrations of P-450 1 (Fig. 4). In order to minimize the contribution of P-450 1 to benzo(a)pyrene metabolism catalyzed by microsomes

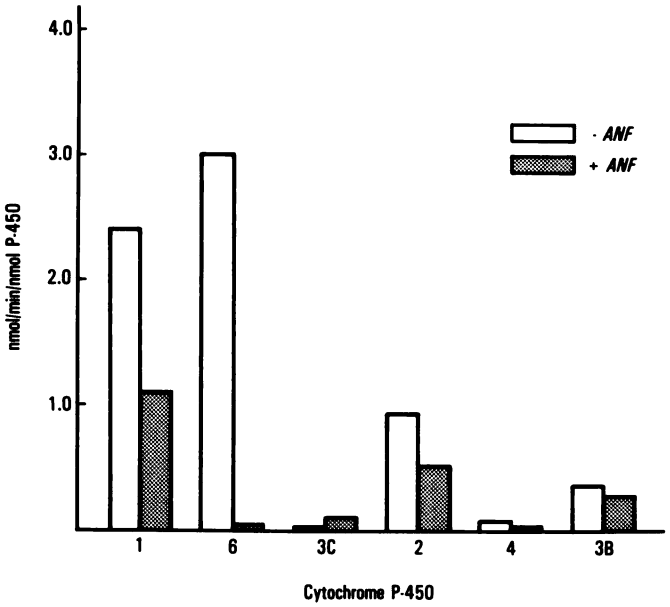

FIG. 3. *Benzo(a)pyrene hydroxylation catalyzed by purified cytochrome P-450 isozymes*

Benzo(a)pyrene hydroxylase activity was determined by a modified method of Van Cantfort using 5 μM [^{14}C]benzo(a)pyrene and 10 μM ANF. Each cytochrome (0.1 nmol) was reconstituted with reductase in the presence of dilauroyl-L-α-lecithin and allowed to incubate 2–5 min prior to addition of the assay buffer. Benzo(a)pyrene hydroxylase activity of the purified cytochromes 1, 6, 3c, 2, 4, and 3b were determined in the absence and presence of ANF.

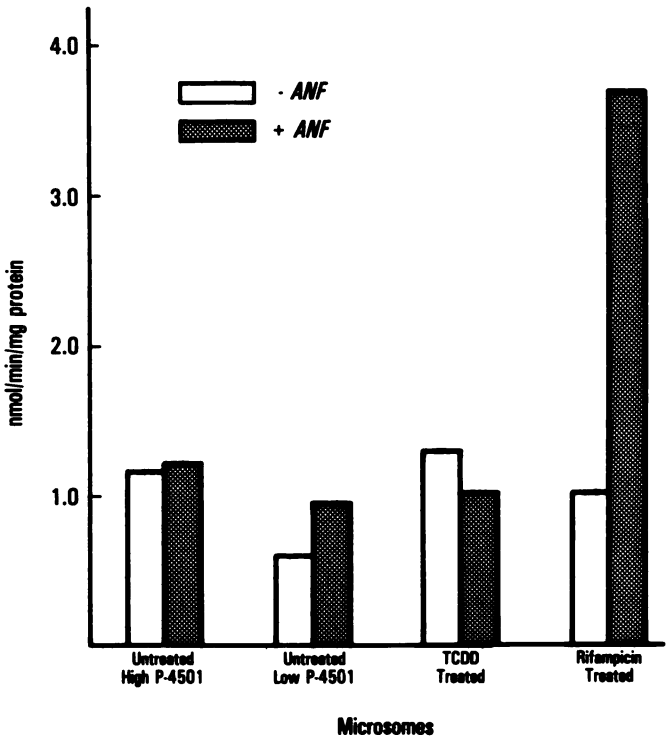

FIG. 4. *Effect of ANF on benzo(a)pyrene hydroxylase activity in microsomes from untreated, TCDD-, and rifampicin-treated rabbits*

Microsomal benzo(a)pyrene hydroxylase activities were determined in the absence and presence of ANF in microsomes from untreated rabbits which contain either a high or low concentration of P-450 1, and microsomes from TCDD- and rifampicin-treated animals. Each value represents the mean rate for preparations from two or more rabbits.

from either TCDD- or rifampicin-treated rabbits, only those microsomes containing low concentrations of P-450 1 were utilized. The effect of these treatments was to increase microsomal benzo(*a*)pyrene activity 2-fold when compared to the basal activity observed in microsomes with low concentrations of P-450 1. In the presence of 10 μM ANF, the activity of liver microsomes from TCDD-treated animals that express low concentrations of P-450 1 and elevated concentrations of P-450 6 is inhibited by 20%. At this concentration of ANF, the activity of P-450 6 should be completely inhibited and the observed activity should reflect the basal activity plus that positively effected by ANF. The observed activity is similar to that observed when microsomes exhibiting basal activity are assayed in the presence of ANF. It is also similar to the effect of ANF on microsomes containing high concentrations of P-450 1 when the catalytic activity of this cytochrome is inhibited by the monoclonal antibody. In contrast, the stimulation by ANF of benzo(*a*)pyrene metabolism is greatly increased when microsomes prepared from rifampicin-treated rabbits are assayed. The percentage increase is more than twice that observed for microsomes exhibiting basal levels of activity and the absolute increase in activity is roughly 7-fold greater, 2.5 versus 0.35 nmol/min/mg.

DISCUSSION

Three benzo(*a*)pyrene hydroxylases that exist in rabbit liver microsomes have been identified by the present investigation. Variations in the expression of any of these three P-450s, which may reflect either genetic variability or the effects of inducing agents, alter the rate of benzo(*a*)pyrene metabolism. In the absence of these effects, microsomes exibit a basal activity of <0.6 nmol/min/mg of protein. Genetic variation leading to expression of elevated concentrations of P-450 1 results in about a 2-fold increase in the overall rate of benzo(*a*)pyrene metabolism. Similar increases in benzo(*a*)pyrene hydroxylase activity are seen following the induction of P-450 6 by TCDD or of P-450 3c by rifampicin. Thus, microsomes containing elevated concentrations of either of the latter three isozymes will exhibit an overall rate of benzo(*a*)pyrene metabolism of about 1.1 nmol/min/mg of protein.

ANF can either stimulate, inhibit, or leave unaffected the activity of individual P-450 enzymes. P-450 6, a TCDD- or polycyclic aromatic hydrocarbon-inducible cytochrome, has been shown to be extensively inhibited by ANF (6, 8). However, it is clear from the results reported here that this flavonoid also inhibits P-450 1, albeit at much higher concentrations. Reconstitution of purified P-450 1 in the presence of 10 μM ANF results in a 50% decrease in benzo(*a*)pyrene hydroxylase activity (Fig. 3). The I_{50} value for P-450 1 is 1000 times higher than that for P-450 6. At 10 μM ANF, P-450 1 is only partially inhibited, however, and in microsomes this inhibition is offset by the stimulation of the activity of other cytochromes. Thus, in the presence of ANF, the net effect is a 10% increase in microsomal benzo(*a*)pyrene hydroxylation. These offsetting processes occur in the presence of both ANF and the antibody to P-450 1 except that, in this case, the antibody inhibits 90–100% of the benzo(*a*)pyrene hydroxylation attributed to P-450 1. The net effect is a rate similar to that seen in the presence of ANF for microsomes from untreated rabbits that contain low concentrations of P-450 1.

The difference in the effect of ANF on microsomes containing high concentrations of P-450 1 and those containing elevated concentrations of P-450 6 reflects the greater extent of inhibition of P-450 6 by ANF. P-450 6 is almost completely inhibited by ANF at this concentration, yielding an activity similar to that produced by ANF with microsomes from untreated rabbits containing low concentrations of P-450 1.

Of the six P-450 enzymes that have been purified and reconstituted, only P-450 3c has been shown (Fig. 3) to be stimulated by ANF (11). Lange *et al.* (21) have suggested that this form of P-450 is induced in rabbit liver by rifampicin. The extent of ANF stimulation of benzo(*a*)pyrene activity is greatly increased following treatment of rabbits with rifampicin, where an increase of 2.7 nmol/min/mg of protein is observed between microsomes assayed in the presence and absence of ANF. This represents a 7-fold increase over the amount of stimulation observed for microsomes prepared from untreated rabbits containing low concentrations of P-450 1 (Fig. 4). The total hepatic concentration of P-450 is increased by 1.5 nmol/mg of protein by treatment with rifampicin. If this is attributed entirely to the induction of P-450 3c, the apparent increase in turnover number affected by ANF can be estimated at about 2 nmol of benzo(*a*)pyrene metabolized/min/nmol P-450 3c, whereas the stimulation observed when the purified enzyme is reconstituted is an order of magnitude lower.

It has been argued (22) that P-450 3c is identical to the cytochrome P-450 B1 isolated by Miki *et al.* (23) who described a requirement for cytochrome b_5 in order to reconstitute this enzyme. The inclusion of cytochrome b_5 in the reconstitution of the enzyme did not, however, alter the rate observed for the metabolism of benzo(*a*)pyrene or the extent of stimulation by ANF. The discrepancy between the results obtained for reconstituted P-450 3c and the microsomal activity may reflect problems with the reconstitution of P-450 3c by standard procedures or the inhibition of the reaction by a contaminant in the cytochrome preparation. In contrast to this incongruity between the reconstituted activity of P-450 3c and the inductive effect of rifampicin, regression analysis of the relation between total benzo(*a*)pyrene hydroxylase activity and microsomal content of P-450 1 yields a value similar to the turnover number for the reconstituted purified enzyme.

When comparing benzo(*a*)pyrene activity of microsomes with a low concentration of P-450 1 to microsomes from TCDD-treated animals, there also appears to be a discrepancy between the results presented here and those of previous investigators. Rabbit liver microsomes from untreated and TCDD-treated animals displayed no differences in activity (24) when the fluorometric assay of Nebert and Gelboin (25) was utilized. This is in contrast to the results presented here in which a 2-fold difference in activity was observed between these two types of

microsomes. Two explanations may be offered for this discrepancy. One is that TCDD effects an increase in the production of nonfluorescent metabolites. Another explanation may be that previous investigators were using microsomes from untreated animals which contained high concentrations of P-450 1.

In conclusion, these results illustrate the problems encountered in determining the outcome of modulation of P-450-mediated carcinogen metabolism. Whether modulation by ANF results in inhibition or stimulation of activity is highly dependent upon the concentration of the modulator and the concentrations of the P-450 enzymes present. Differences in the expression of individual cytochromes, resulting from either exposure of animals to certain xenobiotics or genetic variability, will influence the rate of carcinogen metabolism. This study provides evidence that both induction and genetic variability serve to influence the metabolism of benzo(*a*)pyrene. Treatment of rabbits with rifampicin, which induces an enzyme that can be stimulated by ANF, possibly P-450 3c, or treatment with TCDD, which increases the concentration of P-450 6, results in an increase in the rate of benzo(*a*)pyrene hydroxylation by rabbit liver microsomes. In addition, variability in the concentration of a constitutive cytochrome, P-450 1, which exhibits a high rate of benzo(*a*)pyrene hydroxylase activity also serves to alter the rate of benzo(*a*)pyrene metabolism by microsomes from untreated rabbits. Thus, elevated concentrations of microsomal P-450 1, P-450 3c, or P-450 6 produce an increase in the rate of benzo(*a*)pyrene metabolism, and each of these enzymes is affected differently by ANF.

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