CHANGES IN ARYL HYDROCARBON HYDROXYLASE ACTIVITY AND MICROSOMAL P_{450} DURING POLYCYCLIC HYDROCARBON TREATMENT OF MAMMALIAN CELLS IN CULTURE

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Summary. Benz[a]anthracene in the growth medium stimulated aryl hydrocarbon hydroxylase activity more than 20-fold, while the microsomal P_{450} content of hamster fetal cells was increased less than 2-fold. A blue spectral shift of about 4 nm in the CO-difference absorption peak of cytochrome P_{450} was found in the microsomal fractions of cell cultures exposed to benz[a]anthracene; the appearance of the CO-binding pigment absorbing maximally at a lower wavelength was more closely related to the induced microsomal hydroxylase activity than to the intracellular presence of polycyclic hydrocarbon inducer per se.

Aryl hydrocarbon hydroxylase is an inducible, particulate enzyme system, the properties and kinetics of which have been studied in mammalian fetal cell culture (1, 2). The initial phase of hydroxylase induction by the polycyclic hydrocarbon benz[a]anthracene (BA) dissolved in the growth medium apparently involves synthesis of an induction-specific RNA, and this phase will occur in the presence of BA plus cycloheximide (3). A later phase of enzyme induction presumably involves translation related to this RNA species where the rise in enzyme activity is no longer prevented by actinomycin D, and this phase is independent of the concentration of BA (3). This NADPH-linked hydroxylase system is one of the mixed-function oxygenases having as an active site for their oxidative function the CO-binding cytochrome P_{450}, which has a Soret maximum at about 450 nm upon combination with CO (4, 5). The irreversible conversion of P_{450} to an inactive form, P_{420}, a hemoprotein-CO complex absorbing maximally at about 420 nm (5, 6, 7), is directly related to impairment of benzo[a]pyrene hydroxylation in rat liver fractions (8). Following polycyclic hydrocarbon treatment in vivo (9, 10, 11) or in cell culture (2), there is a blue spectral shift of about 2 to 4 nm in the absorption peak of the P_{450}-CO

complex. I have compared the optical changes of microsomal P_{450} relative to inducible aryl hydrocarbon hydroxylase activity in the presence and absence of cycloheximide. A major manuscript which includes the data presented in this preliminary report has been prepared (12).

Methods

Secondary cultures derived from hamster fetuses of a 10- to 14-day gestational age were used for all experiments. The cell culture materials and techniques were previously described (1). BA and/or cycloheximide in growth medium, or control medium alone, were added to groups of 20 to 50 large (150-mm) tissue culture dishes, between 36 and 72 hours after the secondary cultures had been plated with approximately 0.5 X 10^6 cells/ml. The cells in each group were combined and homogenized in a 0.25 M sucrose-3 mM dithiothreitol solution; the dithiothreitol retarded the conversion of P_{450} to P_{420}, while not interfering with the protoheme, CO-binding pigment and cytochrome b_5 determinations (12). Duplicate determinations of aryl hydrocarbon hydroxylase activity and protein content were performed as previously described (1). The difference spectra of the 78,000 x g pellet fractions were measured in 1-cm cuvettes at room temperature in an Aminco-Chance dual wavelength recording spectrophotometer. The concentrations of total heme (4), cytochrome b_5 (4), the P_{420}-CO complex (5) and cytochrome P_{450} (5) were determined by the methods cited. In the CO-difference spectrum of P_{450}, the optical density at 420 nm is about -45% of the absorption maximum, and my data (12) would indicate that this absorbance below the baseline at 420 nm, and the extinction coefficient, are approximately the same for the polycyclic hydrocarbon-induced form of cytochrome P_{450}.

Results

Following a lag period of about 35 min, 13 μM BA stimulates hydroxylase activity linearly for 12 to 16 hours, the specific activity reaching a maximum during the second 24-hour period (2). If BA-containing medium is replaced with fresh control medium after exposure of the cells to inducer for 20 hours, there is approximately a 2-hour lag period after which the induced hydroxylase

activity decreases with a half-life of 3 to 4 hours (2). During such an experiment on the induction and disappearance of aryl hydrocarbon hydroxylase activity, the microsomal cytochrome content of cells collected at various times was examined. While the hydroxylase activity was stimulated more than 20-fold during a 21-hour exposure to BA, the average total heme, cytochrome b_5 and total CO-binding pigment concentrations all increased less than 2-fold. In cells treated with BA for 21 hours and then grown in fresh control medium alone for 10 more hours, the hydroxylase activity decreased to 40% of its maximally induced specific activity; the microsomal cytochrome content also significantly decreased in these cells. A blue spectral shift in the P_{450}-CO absorption maximum was detectable in microsomes from cells treated with BA for 8 hours, and only the absorbance peak at about 446 nm was evident in cells exposed to inducer for 21 hours. When these cells were again grown in fresh control medium for 10 hours, the 450-nm peak in the CO-difference microsomal spectrum was again visible. There was no difference in the microsomal P_{450}-CO spectral peak between cells exposed to BA for one hour and cells grown in control medium only; however, the entrance of inducer into the cell is virtually complete during the first hour (13).

The question was asked: are the optical changes of cytochrome P_{450} more closely associated with increases in hydroxylase activity, or with the intracellular presence of inducer? Fig. 1 shows the kinetics of aryl hydrocarbon hydroxylase induction during treatment of the cells with an inhibitor of protein synthesis, and following the removal of BA and the inhibitor. In the presence of BA and cycloheximide for 12 hours, there was no significant increase in enzyme activity. However, when the medium containing BA plus cycloheximide was replaced with fresh control medium, approximately a 6-fold increase in hydroxylase activity was observed during the next 10 hours. At the latter point in time, the intracellular content of BA and/or metabolites was less than 2% of that found in the cells after exposure to BA plus cycloheximide for 12 hours (13).

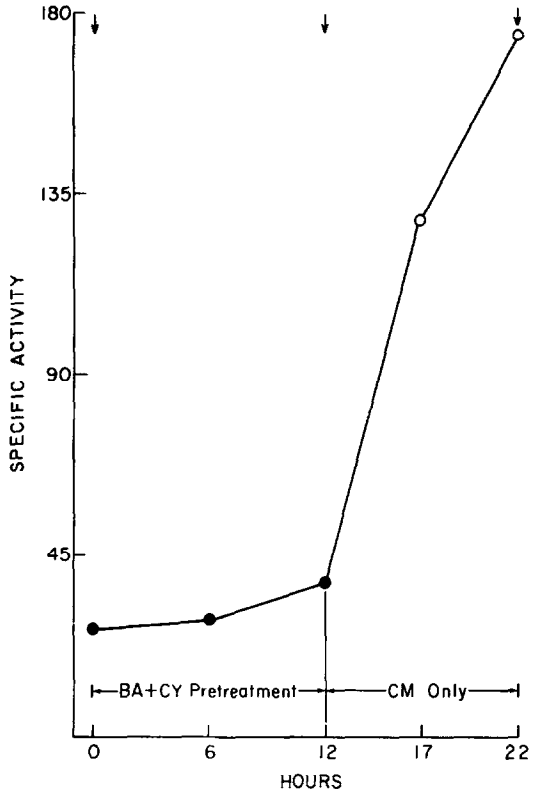

Fig. 1. Induction of aryl hydrocarbon hydroxylase activity in cells grown in fresh control medium (CM), following a 12-hour period in which the cells had been treated with 13 μM cycloheximide (BA + CY). Each point represents duplicate determinations of both enzyme activity and protein on samples of cellular homogenate. The three vertical arrows depict the times at which the cells were harvested for spectrophotometric studies of the microsomes.

Fig. 2 illustrates the CO-difference spectra of the CO-binding cytochrome from cells collected at the three points depicted by arrows in the experiment shown in Fig. 1. The absorbance maximum of the microsomal hemoprotein-CO complex was about 450 nm in cells grown in control medium alone and in cells exposed to inducer plus cycloheximide for 12 hours. However, the CO-binding pigment absorbing maximally at a lower wavelength was detectable in cells where

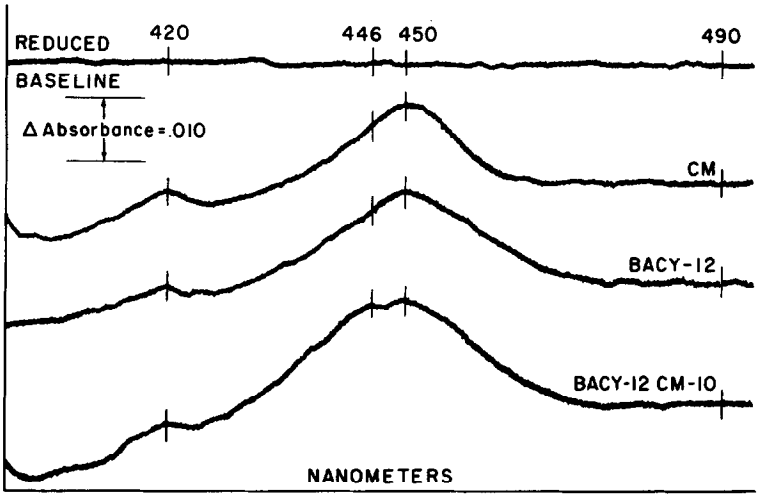

Fig. 2. CO-difference spectra of the three microsomal fractions obtained from the cell culture experiment described in Fig. 1. Protein concentrations of these samples were: 9.3 mg/ml for the reduced baseline and for the fraction from cells grown in control medium only (CM); 10.8 mg/ml for microsomes from cells treated with 13 μM inducer plus 3.5 μM cycloheximide for 12 hours (BACY-12); and 9.1 mg/ml for the microsomal fractions from cells grown for 10 hours in fresh control medium subsequent to the 12-hour exposure to BA plus cycloheximide (BACY-12 CM-10).

the medium containing BA and cycloheximide had been replaced with fresh control medium for 10 hours. In some experiments P_{450}-CO complexes absorbing maximally at about 446 nm and about 450 nm were both distinguishable, while in other experiments these two peaks were not resolved but rather a single absorption peak at about 447 or 448 nm was seen. The degree of spectral resolution appeared to be dependent upon the protein concentration of the turbid sample in the cuvette.

Discussion

These data demonstrate that, in the presence of inducer but with a block of protein synthesis, no blue spectral shift in the microsomal CO-difference

spectrum was found. And, in the relative absence of intracellular inducer but in the presence of increased hydroxylase activity, the CO-binding cytochrome which absorbs maximally at about 446 nm was observed. These findings suggest that the polycyclic hydrocarbon-induced shift of about 4 nm in the CO-difference absorption peak of cytochrome P_{450} is more closely related to the induced microsomal oxygenase activity rather than to the presence of inducer per se in the cell.

Since aryl hydrocarbon hydroxylase activity increased more than 20-fold while the concentration of CO-binding cytochrome increased less than 2-fold, it would appear that neither the control nor the BA-induced P_{450} species is rate-limiting for polycyclic hydrocarbon hydroxylation. These differences in quantitative increases, between hydroxylase activity and the terminal oxidase P_{450} content, suggest that the induction of microsomal oxygenase activity is the result of a more efficient utilization of preexisting components in the oxidation-reduction pathway. In the experiment depicted in Fig. 1, we have suggested (3) that the rise in aryl hydrocarbon hydroxylase activity is due to the translation related to an accumulated induction-specific RNA species. It is therefore postulated that an induction-specific protein may be involved either directly or indirectly in perturbing the ligand field of the iron in the microsomal CO-binding protoheme.

REFERENCES

1. Nebert, D. W., and Gelboin, H. V., J. Biol. Chem. 243, 6242 (1968).
2. Nebert, D. W., and Gelboin, H. V., J. Biol. Chem. 243, 6250 (1968).
3. Nebert, D. W., and Gelboin, H. V., submitted for publication.
4. Omura, T., and Sato, R., J. Biol. Chem. 239, 2370 (1964).
5. Omura, T., and Sato, R., J. Biol. Chem. 239, 2379 (1964).
6. Imai, Y., and Sato, R., European J. Biochem. 1, 419 (1967).
7. Ichikawa, Y., Yamano, T., and Fujishima, H., Biochim, Biophys. Acta 171, 32 (1969).
8. Silverman, D. A., and Talalay, P., Molec. Pharmacol. 3, 90 (1967).
9. Sladek, N. E., and Mannering, G. J., Biochem. Biophys. Res. Commun. 24, 668 (1966).
10. Alvares, A. P., Schilling, G., Levin, W., and Kuntzman, R., J. Pharmacol. Exp. Therap. 163, 417 (1968).
11. Hildebrandt, A., Remmer, H., and Estabrook, R. W., Biochem. Biophys. Res. Commun. 30, 607 (1968).
12. Nebert, D. W., submitted for publication.
13. Nebert, D. W., in preparation.