Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 611-619

DIFFERENTIAL INDUCTION OF RAT HEPATIC CYTOCHROME P-448 AND GLUTATHIONE S-TRANSFERASE B MESSENGER RNAS BY 3-METHYLCHOLANTHRENE

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Received December 10, 1981

Liver poly(A^+)-RNA isolated from untreated and 3-methylcholanthrene treated rats has been translated in the rabbit reticulocyte cell-free system in order to determine the level of translationally active cytochrome P-448, glutathione S-transferase B and serum albumin mRNAs. Translatable cytochrome P-448 mRNA was not detected in untreated rats; however in animals treated with 3-methylcholanthrene cytochrome P-448 mRNA was elevated markedly. Functional rat liver glutathione S-transferase B mRNA was elevated 2-fold by 3-methylcholanthrene administration, whereas the serum albumin mRNA level was decreased by 50%. Our results indicate that 3-methylcholanthrene is not just a specific inducer of drug metabolizing enzymes but can alter the mRNA level encoding other polypeptides and thus affect cellular homeostasis.

INTRODUCTION

Cytochrome P-450 and glutathione S-transferase play a major role in the metabolism of various drugs, mutagens, carcinogens and other foreign compounds. Recent studies from a number of laboratories have demonstrated that both the microsomal cytochrome P-450 and cytosolic glutathione S-transferase are comprised of a family of isozymes with different but overlapping substrate specificities (1-4). As a result the conversion of various xenobiotics to active or inactive metabolites is dependent upon the presence of different forms of cytochrome P-450 and glutathione S-transferase in various tissues.

In order to elucidate the molecular events which lead to an accumulation of rat liver cytochrome P-450 and glutathione S-transferase after xenobiotic administration, we have focused on cytochrome P-448, the major cytochrome P-450 isozyme induced by 3-methylcholanthrene, and glutathione S-transferase B, the predominant glutathione S-transferase isozyme in liver. In this investigation, we have utilized in vitro translation

0006-291X/82/020611-09$01.00/0

and specific immunoprecipitation to quantitate the level of functional cytochrome P-448 and glutathione S-transferase B mRNAs in response to 3-methylcholanthrene administration. Our data indicate that 3-methylcholanthrene administration to rats results in an accumulation of functional mRNAs specific for cytochrome P-448 and glutathione S-transferase B. Surprisingly 3-methylcholanthrene administration resulted in a lowering of rat serum albumin mRNA. Consequently, the increases observed in the amount of immunoprecipitable rat liver cytochrome P-448 and glutathione S-transferase B after 3-methylcholanthrene administration (5-7) can be accounted for by an accumulation of functional mRNAs specific for these proteins.

METHODS

Preparation of Antibodies Against Cytochrome P-448 and Glutathione S-Transferase B

Antibodies against purified cytochrome P-448 and glutathione S-transferase B were raised in New Zealand rabbits. The procedure for antigen purification, antibody preparation and characterization have been presented in detail in previous publications (6,8). Antibody against rat serum albumin was purchased from Cappel Laboratories, Cochranville, PA.

RNA Isolations

Total rat liver RNA was isolated from untreated and 3-methylcholanthrene treated rats by the guanidine thiocyanate method of Chirgwin, et al. (9). Rats treated with 3-methylcholanthrene received a dose of 40 mg/kg body weight and were sacrificed 16 hours later. Poly(A^+)-RNA was isolated from total liver RNA by oligo(dT)-cellulose chromatography.

Cell-Free Protein Synthesis

Rat liver poly(A^+)-RNA was translated for 90 minutes at 30°C in the micrococcal nuclease-treated rabbit reticulocyte lysate. Incubation conditions for translation have been published in detail (10).

Immunoprecipitation of Cytochrome P-448, Glutathione S-Transferase B and Rat Serum Albumin

All in vitro synthesized polypeptides were recovered from the translation mixtures by indirect immunoprecipitation utilizing protein A-sepharose (Pharmacia, Piscataway, NJ). Glutathione S-transferase B was immunoprecipitated as described previously (11). In order to immunoprecipitate cytochrome P-448 and rat serum albumin, translation mixtures were made 2% $NaDodSO_4$, boiled for 3 minutes, and diluted with four volumes of 2.5% Triton X-100, 190 mM NaCl, 50 mM Tris-HCl pH 7.4, and 6 mM EDTA. The mixture was preincubated for 2 hours with 10 µl of preimmune IgG and 100 µl of protein A-sepharose to decrease non-specific binding. The protein A-sepharose slurry was made in 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.05% NP-40 and 0.2% sodium azide. The preimmune IgG/protein A-sepharose complex was removed by centrifugation and to the resultant supernatant 10-15 µl of anti-rat liver cytochrome P-448 or anti-rat liver serum albumin IgG was added. Immediately following the addition of immune IgG, 100 µl of protein A-sepharose was added. The complete mixture was allowed to incubate overnight at 4°C with end to end rotation. Immune complexes bound to protein A-sepharose were recovered by centrifugation and were washed 3-5 times with 0.1% $NaDodSO_4$, 0.5% NP-40 in phosphate buffered saline. Antigen was eluted from the IgG by boiling in $NaDodSO_4$-polyacrylamide gel electrophoresis sample buffer. The

protein A-sepharose was then removed by centrifugation and the supernatant containing the antigen of interest was subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis on 7.5% or 10% gels (12). In order to quantitate the level of translatable cytochrome P-448, glutathione S-transferase B and rat serum albumin mRNAs, the region of the gels containing the immunoprecipitated protein was excised and dissolved in 60% perchloric acid/30% hydrogen peroxide at 60°C. Aquasol-2 was then added to the solubilized gel and total radioactivity was determined by liquid scintillation counting. Background radioactivity was determined by excising gel regions above and below the radiolabeled proteins and dissolving the slices as just described. Values obtained were then subtracted from the radioactivity obtained in the slices containing the radiolabeled proteins.

RESULTS

Cell-Free Translation and Quantitation of Cytochrome P-448 mRNA

Total poly(A^+)-RNA was isolated from untreated rats or from rats treated with 3-methylcholanthrene and translated in the rabbit reticulocyte cell-free system. We found that both poly(A^+)-RNA preparations have very similar translational efficiencies. The incorporation of [^{35}S]-methionine into total protein increased linearly with time and as a function of the concentration of poly(A^+)-RNA added to the lysate. Generally, we observed a decrease in incorporation at RNA concentrations greater than 50 µg/ml. When the translation products directed by poly(A^+)-RNA isolated from untreated and 3-methylcholanthrene treated rats were compared by $NaDodSO_4$-polyacrylamide gel electrophoresis, we observed many differences in the incorporation of [^{35}S]-methionine into various polypeptides (compare lane A with B in Fig. 1).

In order to detect the primary translation product of rat liver cytochrome P-448, the translation mixtures programmed with total liver poly(A^+)-RNA isolated from 3-methylcholanthrene treated rats were subjected to immunoprecipitation with rabbit anti-cytochrome P-448 IgG. The immunoprecipitate obtained from these translation mixtures were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Analysis of the fluorograms of these gels revealed that anti-rat liver cytochrome P-448 IgG selectively immunoprecipitated two polypeptides from the translation system (Fig. 2, lanes B and C). The larger of the two polypeptides co-migrated with purified cytochrome P-448 and has an estimated molecular weight of 55,000 daltons. The lower molecular weight polypeptide has an estimated molecular weight of 54,000 daltons. It appears that these two polypeptides are specific immunoprecipitation products and are induced markedly by 3-methylcholanthrene since neither polypeptide was immunoprecipitated to any great extent from the in vitro translation system programmed with poly(A^+)-RNA

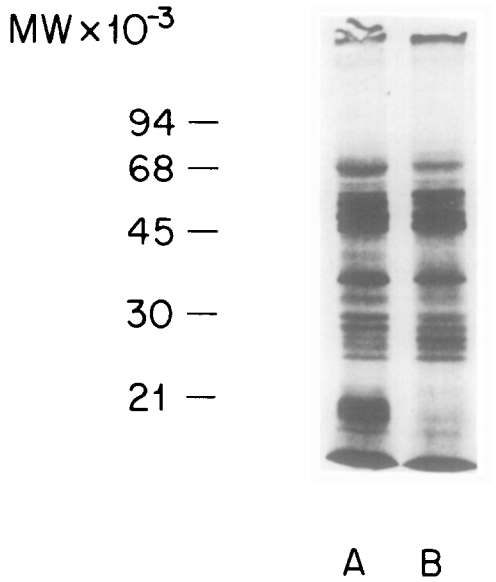

Figure 1 - Autoradiogram of $NaDodSO_4$/polyacrylamide gel electrophoresis of [^{35}S]-methionine-labeled translation products directed by total liver poly(A^+)-RNA isolated from untreated rats (lane A) and 3-methylcholanthrene treated rats (lane B). Equivalent amounts of radioactivity from the translation mixtures programmed with poly(A^+)-RNA were layered on the 10% gel. The molecular weight markers were phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000) and soybean trypsin inhibitor (21,000).

isolated from untreated rats (Fig. 2, lane A). Similarily, when preimmune IgG was used in the place of immune IgG neither of the two polypeptides were observed (data not shown).

Since it was impossible to adequately separate the two polypeptides immunoprecipitated by anti-cytochrome P-448 IgG by $NaDodSO_4$-polyacrylamide gel electrophoresis, we quantitated the level of functional liver cytochrome P-448 mRNA by excising the region of the gel containing the two radiolabeled polypeptides and comparing the amount of radioactivity incorporated into these polypeptides as a function of 3-methylcholanthrene induction. The level of functional cytochrome P-448 mRNA in untreated rats (Table I and Fig. 2, lane A) is below the level of accurate detection and quantitation by cell-free translation. However in rats treated with 3-methylcholanthrene, the amount of immunoreactive cytochrome P-448 is markedly elevated (Table I). In addition it appears from analyzing the fluorograms that the amount of [^{35}S]-methionine incorporated into these two polypeptides is evenly distributed between them.

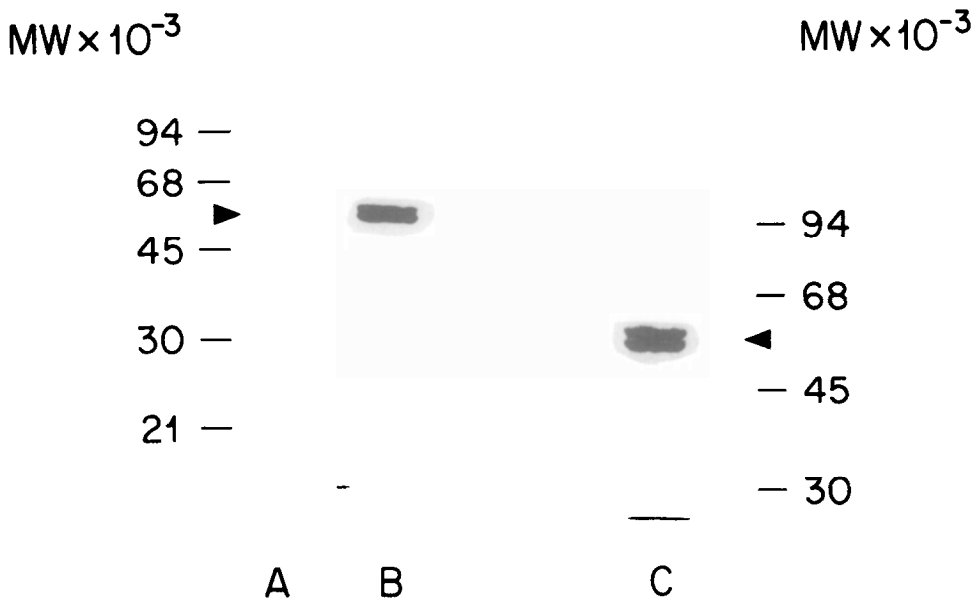

Figure 2 - Fluorogram of NaDodSO$_4$/polyacrylamide gel electrophoresis of cytochrome P-448 immunoprecipitated from rabbit reticulocyte lysate translation mixtures programmed with poly(A$^+$)-RNA isolated from untreated rats (lane A) and 3-methylcholanthrene treated rats (lanes B and C). Equal aliquots of the immunoprecipitated cytochrome P-448 were layered on either 10% gels (lanes A and B) or 7.5% gels (lane C). The arrowhead denotes the position of purified cytochrome P-448 in the Coomassie Brilliant Blue stained gels from which the fluorograms were made. The molecular weight markers are identical to those described in Fig. 1.

Cell-Free Translation and Quantitation of Rat Liver Glutathione S-Transferase B and Serum Albumin mRNAs

Glutathione S-transferase B and serum albumin mRNAs were quantitated as described above for cytochrome P-448. The primary translation product of glutathione S-transferase B consisted of two polypeptides with molecular weights of 29,000 and 27,000. These two in vitro synthesized polypeptides comigrated exactly with the purified polypeptide subunits (Fig. 3, lanes A and B). Treatment of rats with 3-methylcholanthrene results in a 2-fold induction of glutathione S-transferase B mRNA (Table I). As we observed previously with phenobarbital (11), the elevation in glutathione S-transferase B mRNA appears to be specific for the mRNA encoding the small molecular weight subunit rather than both subunits (Fig. 3, compare lane A with B). In contrast to glutathione S-transferase B mRNA levels, the rat serum albumin mRNA level was decreased approximately 50% in 3-methylcholanthrene treated rats (Table I and Fig. 3, lanes C and D). Although this was a surprising finding, it indicates that 3-methylcholanthrene is not just a specific inducer of mRNAs encoding drug metabolizing

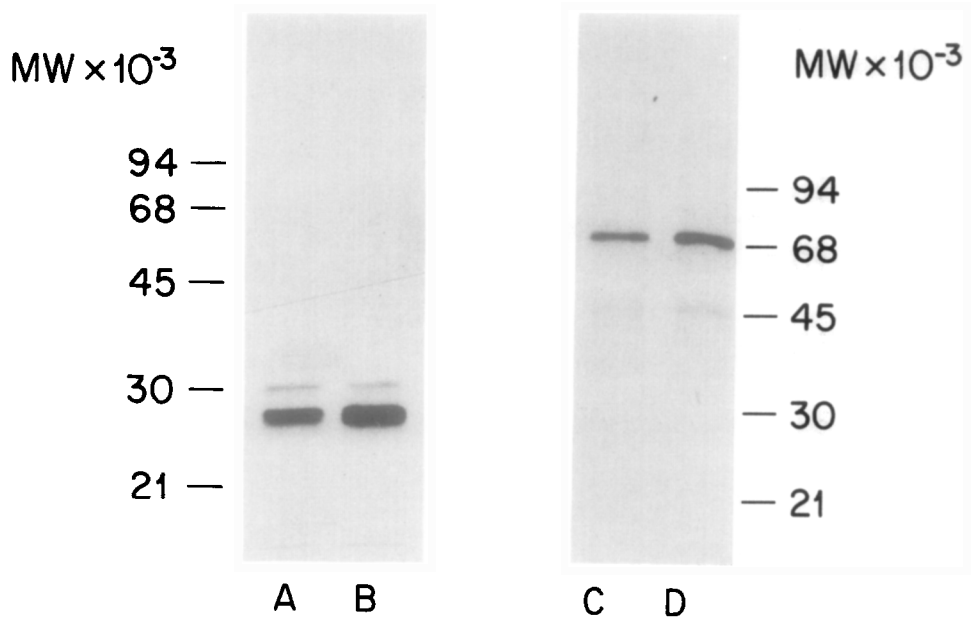

Figure 3 - Fluorogram of NaDodSO$_4$/polyacrylamide gel electrophoresis using 10% gels of immunoprecipitated rat liver glutathione S-transferase B and preproalbumin. Lane A represents glutathione S-transferase B immunoprecipitated from the rabbit reticulocyte lysate programmed with poly(A$^+$)-RNA isolated from untreated rats while lane B represents glutathione S-transferase B immunoprecipitated from the lysate programmed with poly(A$^+$)-RNA isolated from 3-methylcholanthrene treated rats. Lane D represents preproalbumin immunoprecipitated from the lysate programmed with poly(A$^+$)-RNA isolated from untreated rats while lane C represents preproalbumin immunoprecipitated from translation systems programmed with poly(A$^+$)-RNA isolated from 3-methylcholanthrene-treated rats. The molecular weight markers are identical to those described in Figure 1.

TABLE I

Quantification of Functional Rat Liver Cytochrome P-448, Glutathione S-Transferase B and Serum Albumin mRNAs in Response to 3-Methylcholanthrene Administration

	Treatment	cpm in Immunoprecipitated Cytochrome P-448 x 10^{-4}		cpm in Immunoprecipitated Glutathione S-Transferase B x 10^{-4}		cpm in Immunoprecipitated Rat Serum Albumin x 10^{-5}	
Exp. 1	None	N.D.[a]	(5.63)[b]	2.41	(12.99)	1.41	(6.33)
	3-Methylcholanthrene	1.36		4.18		0.73	
Exp. 2	None	N.D.	(9.32)	1.42	(6.15)	1.05	(7.83)
	3-Methylcholanthrene	2.97		2.67		0.53	

a - N.D. (not determined). The level of cytochrome P-448 mRNA was below the limit of detection and quantitation by the translation assay.

b - The values in parenthesis represent the amount of trichloroacetic acid precipitable radioactivity utilized in each immunoprecipitation reaction. The values are cpm x 10^{-6}.

enzymes but can affect the level of mRNAs encoding for polypeptides unrelated to drug metabolism.

DISCUSSION

In this investigation we have quantitated the level of functional mRNAs specific for rat liver cytochrome P-448, glutathione S-transferase B and serum albumin and have demonstrated that 3-methylcholanthrene alters the mRNA level for all three of these proteins. Cytochrome P-448 and glutathione S-transferase B mRNAs are induced by 3-methylcholanthrene, whereas the albumin mRNA level is decreased by 50%. Similar results have been reported recently on the induction of rat liver cytochrome P-448 by 3-methylcholanthrene by Bresnick, et al. (13) and on the induction of mouse liver cytochrome P_1-450 by Negishi and Nerbert (14). Our results indicate that 3-methylcholanthrene does not act in a concerted fashion and is not a specific effector of drug metabolizing enzymes. In addition, the extent of induction we observe in functional mRNA levels for cytochrome P-448 and glutathione S-transferase B are in agreement with data obtained by immunological quantitation of these proteins as a function of 3-methylcholanthrene induction (5-7). Consequently, the elevation in immunoreactive cytochrome P-448 and glutathione S-transferase B can be totally accounted for by similar increases in the level of functional mRNA encoding these proteins.

Kumar and Padmanaban (15) have recently suggested that cytochrome P-448 is synthesized initially as a high molecular weight precursor (M_r = 59,000) and is processed to the mature enzyme (M_r = 55,000) in the presence of microsomal membranes. The data we obtained in our study do not agree with those of Kumar and Padmanaban. We find no evidence for the existence of a high molecular weight precursor. This latter finding is in agreement with other studies (13,16). However, we do observe that the antibody used in this study recognizes two in vitro synthesized polypeptides both of which are induced by 3-methylcholanthrene. One of the polypeptides comigrates with purified cytochrome P-448 (M_r~55,000) and the other migrates with a M_r of 54,000. The relationship between these two polypeptides remains to be elucidated.

Our results with glutathione S-transferase B support our earlier finding (11) and suggests that the subunits may arise from two distinct mRNAs rather than post translational processing of the large subunit to the small subunit (17). As with the

case with phenobarbital, 3-methylcholantrene appears to elevate the mRNA encoding only the small molecular weight subunit. This subunit has been demonstrated to possess both glutathione S-transferase conjugating activity and a high affinity site which participates in the non-covalent binding of several non-substrate ligands which include bilirubin, steroids, and heme (18). It is possible that this subunit might play a regulatory role in heme binding and transport during phenobarbital or 3-methylcholanthrene induction of cytochrome P-450 isozymes. Although the decrease in rat serum albumin mRNA level was surprising, it illustrates that 3-methylcholanthrene is not just an inducer of drug metabolizing enzymes. It clearly has effects on mRNA levels encoding other proteins and can consequently alter cellular homeostasis.

ACKNOWLEDGMENTS

We would like to thank Joan Kiliyanski for help in preparing this manuscript.

REFERENCES

1. Guengerich, F.P. (1979) Pharmacol. Ther. Part A Chemother. Toxicol. Metab. Inhibitors 6, 99-121.

2. Johnson, E.F. (1979) Reviews in Biochemical Toxicology, Vol. 1 (Hodgson, E., Bend, J.R. and Philipot, R.M., eds), pp. 1-26, Elsevier/North Holland, NY.

3. Jakoby, W.B. (1978) Adv. Enzymology 46, 383-414.

4. Lu, A.Y.H. and West, S.B. (1980) Pharmacol. Rev. 31, 277-295.

5. Thomas, P.E., Reik, L.M., Ryan, D.E. and Levin, W. (1981) J. Biol. Chem. 256, 1044-1052.

6. Pickett, C.B., Jeter, R.L., Morin, J. and Lu, A.Y.H. (1981) J. Biol. Chem. 256, 8815-8820.

7. Hales, B.F. and Neims, A.H. (1977) Biochem. Pharmacol. 26, 555-556.

8. Hales, B.F. and Neims, A.H. (1976) Biochemical J. 160, 223-229.

9. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.

10. Pickett, C.B. and Lu, A.Y.H. (1981) Proc. Natl. Acad. Sci. USA 78, 893-897.

11. Pickett, C.B., Wells, W., Lu, A.Y.H. and Hales, B.F. (1981) Biochem. Biophys. Res. Commun. 99, 1002-1010.

12. Laemmli, U.K. (1970) Nature (London) 227, 680-685.

13. Bresnick, E., Brosseau, M., Levin, W., Reik, L., Ryan, D.E. and Thomas, P.E. (1981) Proc. Natl. Acad. Sci. USA 78, 4083-4987.

14. Negishi, M. and Nebert, D.W. (1981) J. Biol. Chem. 256, 3085-3091.

15. Kumar, A. and Padmanaban, G. (1980) J. Biol. Chem. 255, 522-525.

16. Dubois, R.N., John, M.E., Waterman, M.R., West, S.B. and Lu, A.Y.H. (1980) in Biochemistry, Biophysics and Regulation of Cytochrome P-450 (Gustafasson, J.-A., Carlstedt-Duke, J., Mode, A and Raftery, J., eds), pp. 397-404, Elsevier-North Holland Publishing Co., New York.

17. Bhargava, M.M., Ohmi, N., Listowsky, I. and Arias, I.M. (1980) J. Biol. Chem. 255, 718-723.

18. Arias, I.M., Fleischner, G., Kirsch, R., Mishkin, S. and Gatmaitan, Z. (1976) in Glutathione: Metabolism and Function (Arias, I.M. and Jakoby, W.B., eds), pp. 175-188, Raven Press, New York.