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MOLECULAR REGULATION OF ETHANOL-INDUCIBLE CYTOCHROME P450-IIE1 IN HAMSTERS*

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SUMMARY: Liver polysomal poly(A)+ RNA, isolated from hamsters treated with ethanol or pyrazole, was translated in vitro to determine the effect of these compounds on specific mRNA encoding P450-IIE1, an ethanol-inducible P450 isozyme. As assessed by immunoprecipitation of translation products, ethanol and pyrazole increased hepatic P450-IIE1 mRNA levels by 160% and 45%, respectively, when compared to controls. In liver microsomes from the same animals, ethanol and pyrazole caused a two-fold increase in microsomal P450-IIE1 protein and a two- to three-fold enhancement of microsomal ethanol oxidation and p-nitrophenol hydroxylation. Our results show that the induction of P450-IIE1 protein in hamsters by ethanol and pyrazole, an "ethanol-like" inducer, is accompanied by an increase in translatable P450-IIE1 mRNA.

Cytochrome P450-IIE1# is an isozymic form of microsomal P450 that is inducible by ethanol and other xenobiotics of diverse structure (acetone, isoniazid, imidazole, pyrazole, etc) (1-3). Among the various catalytic properties of this hemeprotein, which has been purified from rodents (4-6) and from humans (7,8), are ethanol oxidation, acetaminophen oxidation, N-nitrosodimethylamine demethylation and carbon tetrachloride oxidation (3,4,8-12). Since P450-IIE1 may play a key role in the toxicity of such agents, particularly after chronic alcohol consumption (13), the molecular mechanism(s) by which microsomal P450-IIE1 levels are regulated is of considerable importance. Previous studies have shown that the induction of other P450 isozymes by compounds such as phenobarbital, 3-methylcholanthrene or pregnenolone-16α-carbonitrile is mediated by an increase in their corresponding mRNAs (14-18), resulting primarily from transcriptional activation of P450

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#According to the newly-recommended nomenclature for P450 isozymes (29), we have designated the hamster liver P450 isozyme inducible by ethanol as P450-IIE1.

Abbreviation used: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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structural genes. In contrast, recent reports have concluded that P450-IIE1 protein induction by certain "ethanol-like" agents may be regulated by a post-translational event rather than via increased P450-IIE1 gene transcription; neither study found, using P450-IIE1 cDNA probes, an accumulation of mRNA transcripts (19,20). In this report, we describe our studies concerning the mechanism of P450-IIE1 induction by ethanol and "ethanol-like" compounds using another approach, namely, by quantitating translatable P450-IIE1 mRNA levels.

MATERIALS AND METHODS

Animals

Male Syrian golden hamsters (80-100 g body weight), purchased from Charles River Breeding Laboratories (Wilmington, MA), were maintained with food and water ad libitum. Ethanol was administered as a 10% solution in drinking water for 10 days until animals were killed while pyrazole-treated animals were given a single intraperitoneal injection (200 mg/kg) and killed 8 hr later. One-half of the liver (2 g) was used for RNA isolation while the remainder was used to prepare microsomes.

Poly(A)$^+$ RNA Preparation

Hepatic polysomes were prepared according to Shapiro and Young (21). Polysomal pellets were obtained from the post-mitochondrial fraction by ultracentrifugation (175,000 x g for 2 hr) through a 1 M sucrose cushion and resuspended in 25 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 5 mM $MgCl_2$, 0.1% Lubrol PX, 1 µg cycloheximide/ml and 20 units heparin/ml. Chromatography on Oligo(dT)-cellulose (type 2, Collaborative Research, Bedford, MA) was then used to purify the poly(A)$^+$ RNA fraction (22).

In Vitro Translation Assays

Hepatic poly(A)$^+$ RNA (2 µg) was translated in vitro using a nuclease-treated rabbit reticulocyte lysate system (Promega Biotec, Madison, WI) according to the manufacturer's protocol. [^{35}S]methionine (1100 Ci/mmol; NEN Research Products, Boston, MA) was used as the radioactive precursor (0.5 µCi/ml). Specific translation products were immunoprecipitated from total translation products with anti-hamster P450-IIE1 IgG together with Protein A-Sepharose CL-4B (15). [^{35}S]methionine-incorporated radioactivity was determined by scintillation counting of trichloroacetic acid-precipitable protein on glass fiber filters (Whatman GF-C). P450-IIE1 mRNA content was assessed from the ratio of immunoprecipitable radioactivity versus the radioactivity in total translation products (14).

Other Methods

Liver microsomes were prepared using conventional differential centrifugation techniques. For P450-IIE1 immunoquantitation, microsomal proteins were first separated by SDS-PAGE (23), transferred to nitrocellulose, and then immunochemically-stained with anti-hamster P450-IIE1 IgG (6). Immunoreaction intensity was determined by computerized scanning densitometry (Biomed Instruments Inc., Fullerton, CA). Ethanol oxidation by liver microsomes was determined by measurement of acetaldehyde formation using a head-space gas chromatographic method (24) while benzphetamine N-demethylation and p-nitrophenol hydroxylation were measured using colorimetric assays for formaldehyde (25) and 4-nitrocatechol formation (26), respectively.

RESULTS

Polyclonal antibodies directed against hamster P450-IIE1 recognized a single major protein (Mr=54,000) in hamster liver microsomes, the immunochemical-staining intensity of which increased significantly after ethanol or pyrazole treatment (Figure 1a). The electrophoretic mobility of this protein, P450-IIE1, relative to other hamster liver microsomal proteins and its induction following ethanol or pyrazole administration is also shown in the conventionally-stained gel (Figure 1b). Reaction of anti-hamster P450-IIE1 IgG with total polysomal poly(A)$^+$ RNA-derived translation products (Figure 2, lane 2) resulted in immunoprecipitation of a radiolabelled polypeptide with the same molecular weight as authentic hamster P450-IIE1 (Figure 2, lane 3). The fluorographic intensity of this immunoprecipitable protein was much stronger when liver poly(A)$^+$ RNA derived from ethanol- or pyrazole-treated animals was

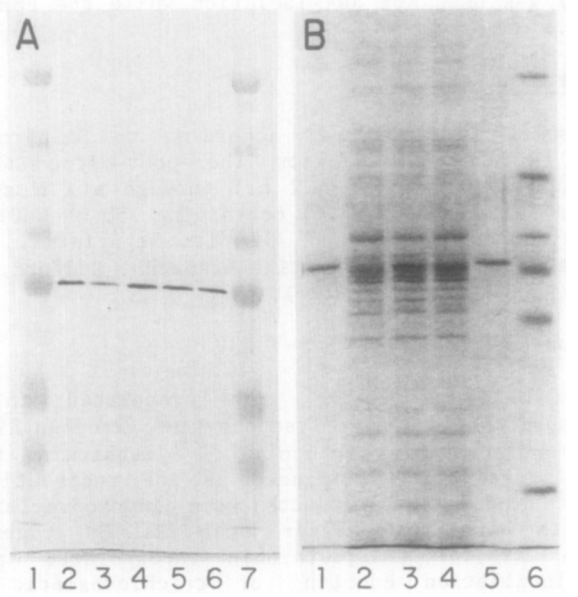

Figure 1. Conventional SDS-PAGE and immunoblot analysis of hamster liver microsomes. Panel A. Proteins resolved by SDS-PAGE (conditions described in legend to Panel B) were electrophoretically transferred to nitrocellulose, and then immunochemically-stained with anti-hamster P450-IIE1 IgG. Lanes 3, 4 and 5, liver microsomes (0.5 µg) from untreated, ethanol-treated and pyrazole-treated hamsters, respectively; lanes 2 and 6, purified hamster P450-IIE1 (0.025 µg); lanes 1 and 7, prestained protein standards with molecular weights of 116,000, 84,000, 58,000, 49,000, 36,000 and 27,000 (2 µg each, top to bottom). Panel B. Microsomes were subjected to electrophoresis on a slab gel (0.75 mm thick) containing 7.5% acrylamide. Proteins were visualized by Coomassie Blue R-250 staining. Lanes 1 and 5, purified hamster P450-IIE1 (0.25 µg); lanes 2, 3 and 4, liver microsomes (2 µg) from untreated, ethanol-treated, and pyrazole-treated hamsters, respectively; lane 6, protein standards with molecular weights of 98,000, 68,000, 58,000, 53,000, 48,000 and 29,000 (0.5 µg each, top to bottom).

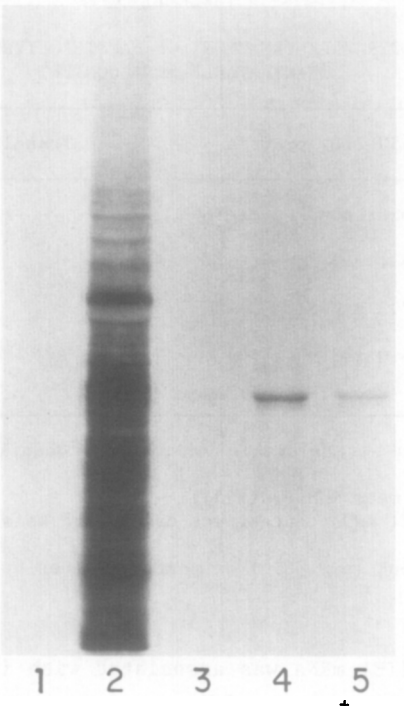

Figure 2. Electrophoretic analysis of hamster poly(A)$^+$ RNA-dependent translation products. Hepatic poly(A)$^+$ RNA (2 µg) was translated in vitro using a rabbit reticulocyte lysate system. Translation products were immunoprecipitated with anti-hamster P450-IIE1 IgG, subjected to SDS-PAGE, and visualized by fluorography. Migration proceeds from top to bottom. Lanes 3, 4 and 5, immunoprecipitates of translation products (2×10^6 dpm) from untreated, ethanol-treated and pyrazole-treated hamsters, respectively; lane 1, immunoprecipitate of a blank translation (RNA omitted); lane 2, total translation products (2×10^5 dpm).

used to program the translation reaction (Figure 2, lanes 4 and 5).

Quantitative data obtained in experiments similar to those illustrated are summarized in Table I. Ethanol treatment caused a 160% increase in translatable P450-IIE1 mRNA when compared to control levels while pyrazole treatment caused a 45% increase. Immunoquantitation of microsomal P450-IIE1 protein revealed a two-fold increase in specific content after either treatment. This increase in microsomal P450-IIE1 content was reflected by enhanced rates of ethanol oxidation and p-nitrophenol hydroxylation (two drug-metabolizing activities catalyzed by P450-IIE1) in microsomes from animals administered ethanol or pyrazole whereas benzphetamine N-demethylation was not affected by either treatment (Table II).

DISCUSSION

In this report, we have shown an increase in translatable hepatic P450-IIE1 mRNA in hamsters following treatment with either ethanol or pyrazole. This

TABLE I

EFFECT OF ETHANOL AND PYRAZOLE TREATMENT ON HAMSTER LIVER P450-IIE1 PROTEIN AND TRANSLATABLE mRNA CONTENT

Treatment	P450-IIE1 protein[a]	Translatable P450-IIE1 mRNA[a,b]
	μg/mg microsomal protein	%
None	29.1 ± 3.2	0.28 ± 0.03
Ethanol	57.3 ± 3.5[c]	0.73 ± 0.16[c]
Pyrazole	56.0 ± 3.9[c]	0.40 ± 0.07

P450-IIE1 protein and translatable mRNA were quantitated as described in Materials and Methods.
[a]Values represent the mean ± S.D. (n=3).
[b]Translatable P450-IIE1 mRNA content was calculated as a percentage of the total translatable mRNA.
[c]Significantly different ($p<0.05$) from control values by a Newman-Keuls' post-hoc test (30).

increase in liver P450-IIE1 mRNA was associated with increased liver microsomal P450-IIE1 protein content and enhanced rates of microsomal ethanol oxidation and p-nitrophenol hydroxylation. Quantitatively, the increase in translatable P450-IIE1 mRNA levels after ethanol treatment closely paralleled the elevation of P450-IIE1 protein and associated catalytic activities whereas the

TABLE II

CATALYTIC ACTIVITIES OF LIVER MICROSOMES FROM HAMSTERS TREATED WITH ETHANOL OR PYRAZOLE

Treatment	Ethanol Oxidation	p-Nitrophenol Hydroxylation	Benzphetamine N-demethylation
	nmol product formed/min/mg protein[a]		
None	8.8 ± 0.6	3.2 ± 0.4	14.8 ± 0.8
Ethanol	19.4 ± 2.2[b]	9.1 ± 0.9[b]	17.0 ± 3.1
Pyrazole	18.1 ± 1.0[b]	8.5 ± 0.9[b]	15.1 ± 0.8

Incubation mixtures contained 100 mM potassium phosphate buffer (pH 7.0 or 7.4), 100-130 μg microsomal protein, substrate (50 mM ethanol, 0.1 mM p-nitrophenol or 1 mM benzphetamine) and 1 mM NADPH in a final volume of 1.0 ml. Reactions were initiated with NADPH and terminated after 10 min at 37°C. Using these conditions, product formation (determined as described in Materials and Methods) was directly proportional to both protein concentration and time of incubation.
[a]Values represent the mean ± S.D. (n=3).
[b]Significantly different ($p<0.05$) from control values by a Newman-Keuls' post-hoc test.

pyrazole-mediated increase in mRNA was less than the hemeprotein and catalytic activity increases (Table I). In rabbits, a transient increase in P450-IIE1 mRNA was noted only 3 hr after imidazole administration (20). When considered together with the proposed extremely rapid turnover of P450-IIE1 protein (27), the time elapsed between parenteral administration of P450-IIE1 inducers and RNA preparation may be critical for determining the full extent of P450-IIE1 mRNA increases. Since hamsters treated with ethanol are constantly imbibing this inducing agent (24), both P450-IIE1 mRNA and protein levels may be in a new steady-state of increased synthesis/degradation. In contrast, pyrazole-treated hamsters were killed 8 hr after drug administration, a time point when the enzyme was already fully-induced but the encoding mRNA was probably decreasing towards control levels. Time course studies assessing hepatic P450-IIE1 mRNA content after pyrazole treatment are needed to confirm this.

Our results concerning increased liver P450-IIE1 mRNA in ethanol- and pyrazole-treated hamsters contrast with a previous report. Song et al. (19) found no increase in hepatic P450-IIE1 mRNA in rats after treatment with pyrazole, 4-methylpyrazole or acetone (the latter given orally) but described a 4-fold increase in both microsomal P450-IIE1 protein and aniline hydroxylase activity. Since animals were killed 24 hr after pyrazole and 4-methylpyrazole administration (the treatment protocol with acetone was not fully-described), it is possible that P450-IIE1 mRNA levels had already returned to control values. In a study by these same researchers concerning the effect of starvation (a treatment that induces P450-IIE1 protein) on P450-IIE1 mRNA, rats killed after a 48 hr fast showed a 250% elevation in mRNA levels (28). Although species and/or methodology differences cannot be ruled out, we believe that increases in microsomal P450-IIE1 observed after treatment with ethanol or "ethanol-like" inducers are due, at least in part, to increases in the mRNA encoding this enzyme. It remains to be established whether such increases in P450-IIE1 messenger result from transcriptional activation of the P450-IIE1 gene or mRNA stabilization.

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REFERENCES

1. Ohnishi, K. and Lieber, C.S. (1977) J. Biol. Chem. 252, 7124-7131.
2. Koop, D.R., Crump, B.L., Nordblom, G.D. and Coon, M.J. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4065-4069.
3. Thomas, P.E., Bandiera, S., Maines, S.L., Ryan, D.E. and Levin, W. (1987) Biochemistry 26, 2280-2289.
4. Koop, D.R., Morgan, E.T., Tarr, G.E. and Coon, M.J. (1982) J. Biol. Chem. 257, 8472-8480.

5. Ryan, D.E., Ramanathan, L., Iida, S., Thomas, P.E., Haniu, M., Shively, J.E., Lieber, C.S. and Levin, W. (1985) J. Biol. Chem. 260, 6385-6393.
6. Lasker, J.M., Ardies, C.M., Bloswick, B.P. and Lieber, C.S. (1986) Fed. Proc. 45, 1655.
7. Wrighton, S.A., Thomas, P.E., Molowa, D.T., Haniu, M., Shively, J.E., Maines, S.L., Watkins, P.B., Parker, G., Mendez-Picon, G., Levin, W. and Guzelian, P.S. (1986) Biochemistry 25, 6731-6735.
8. Lasker, J.M., Raucy, J., Kubota, S., Bloswick, B.P., Black, M. and Lieber, C.S. (1987) Biochem. Biophys. Res. Comm. (in press).
9. Morgan, E.T., Koop, D.R. and Coon, M.J. (1983) Biochem. Biophys. Res. Comm. 112, 8-13.
10. Yang, C.S., Tu, Y.Y., Koop, D.R. and Coon, M.J. (1985) Cancer Res. 45, 1140-1145.
11. Levin, W., Thomas, P.E., Oldfield, N. and Ryan, D.E. (1986) Arch. Biochem. Biophys. 248, 158-165.
12. Johansson, I. and Ingelman-Sundberg, M. (1985) FEBS Lett. 183, 265-269.
13. Lieber, C.S. (1982) In: Medical Disorders of Alcoholism: Pathogenesis and Treatment. W.B. Saunders, Philadelphia, pp. 259-312.
14. Phillips, I.R., Shephard, E.A., Mitani, F. and Rabin, B.R. (1981) Biochem. J. 196. 839-851.
15. Pickett, C.B., Jeter, R.L., Wang, R. and Lu, A.Y.H. (1983) Arch. Biochem. Biophys. 225, 854-860.
16. Hardwick, J.P., Gonzalez, F.J. and Kasper, C.B. (1983) J. Biol. Chem. 258, 8081-8085.
17. Kawajiri, K., Gotoh, O., Tagashira, Y., Sogawa, K. and Fujii-Kuriyama, Y. (1984) J. Biol. Chem. 259, 10145-10149.
18. Hardwick, J.P., Gonzalez, F.J. and Kasper, C.B. (1983) J. Biol. Chem. 258, 10182-10186.
19. Song, B.-J., Gelboin, H.V., Park, S.-S., Yang, C.S. and Gonzalez, F.J. (1986) J. Biol. Chem. 261, 16689-16697.
20. Khani, S.C., Zaphiropolos, P.G., Fujita, V.S., Porter, T.D.,Koop, D.R. and Coon, M.J. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 638-642.
21. Shapiro, S.Z. and Young, J.R. (1981) J. Biol. Chem. 256, 1495-1498.
22. Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412.
23. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
24. Ardies, C.M., Lasker, J.M. and Lieber, C.S. (1987) Biochem. Pharmacol. (in press).
25. Nash, T. (1953) Biochem. J. 55, 416-421.
26. Koop, D.R. (1986) Mol. Pharmacol. 29, 399-404.
27. Hetu, C. and Joly, J.G. (1985) Biochem. Pharmacol. 34, 1211-1216.
28. Hong, J., Pan, J., Gonzalez, F.J., Gelboin, H.V. and Yang, C.S. (1987) Biochem. Biophys. Res. Comm. 142, 1077-1083.
29. Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Phillips, I.R., Sato, R. and Waterman, M.R. (1987) DNA 6, 1-11.
30. Winer, B.J. (1971) In: Statistical Principles in Experimental Design (2nd ed.) McGraw-Hill, New York.