Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 37-42

HORMONE CONTROLLED PHOSPHORYLATION AND DEGRADATION OF CYP2B1 AND CYP2E1 IN ISOLATED RAT HEPATOCYTES

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Received November 16, 1990

Addition of adrenalin to primary rat hepatocytes caused a 3- and 2-fold increase in [^{32}P]-incorporation into CYP2E1 and CYP2B1, respectively. Adrenalin also increased the rate of CYP2E1 degradation at similar concentrations as needed for phosphorylation of the protein (r=0.93), but did not influence the degradation rate of CYP2B1. Ethanol (75 mM) completely protected from adrenalin dependent phosphorylation and degradation of CYP2E1, but did not influence CYP2B1 on these parameters. Examination of *para*-nitrophenol hydroxylase revealed that ethanol stabilized the catalytically active form of CYP2E1. Insulin treatment caused a stabilization of CYP2E1, but did not affect CYP2B1 degradation. It is concluded that degradation of CYP2E1 is the subject of hormonal control, whereas CYP2B1 decomposition is accomplished in a different and a less regulated manner.

The hepatic microsomal monooxygenase system is adaptable to changes in the environment. Thus, many of the various forms of cytochromes P450 are inducible by their own substrates. It appears that the P450 substrates or other inducers can influence the rate of specific gene transcription (1, 2), the stability of the specific mRNA (3), the rate of protein translation (4) or the stability of the enzyme (5-8).

We have recently described that specific ligands to CYP2E1# can protect the enzyme from degradation in hepatocyte cultures (5, 6). This effect is achieved at concentrations which parallels the binding affinity of the ligands to the enzyme (5). The CYP2E1 substrates also protect the enzyme from a glucagon and cAMP-dependent phosphorylation on Ser^{129}, a reaction that under *in vitro* conditions causes heme loss. The

#The nomenclature for cytochrome P450 used is as proposed in Nebert, D.W., Gonzalez, F.J., Coon, M.J., Estabrook, R.W., Feyereisen, R., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Loper, J.C., Nelson, D.R., Sato, R., Waterman, M. and Waxman, D.J. (1991). DNA Cell Biol, in press.

0006-291X/91 $1.50

phosphorylated enzyme is then apparently degraded at a higher rate than the noncovalently modified protein (6).

In the present investigation, we describe that adrenalin stimulation of hepatocytes causes phosphorylation of both CYP2E1 and CYP2B1, but only affects CYP2E1 degradation, whereas insulin has an opposite effect regarding CYP2E1 degradation.

EXPERIMENTAL PROCEDURES

Materials. Collagenase (type IV, lot 58F-6832), adrenalin (lot 119C-05811) were obtained from Sigma. Porcine Sodium Insulin (Lot 050487) was purchased from Lilly. Antisera against CYP2E1 and CYP2B1 were prepared as previously described (9) and have been characterized before (9, 10).

Methods. Hepatocytes were prepared from male Sprague Dawley rats (170 g), previously starved and treated with acetone (5 ml/kg) for two days. This treatment is known to increase the hepatic level of CYP2E1 about 10-fold to approximately 0.4 nmol/mg and cause induction of CYP2B1 to about 0.7 nmol/mg of microsomal protein (9). Culturing of hepatocytes from starved and acetone treated rats on collagen rapidly causes degradation of CYP2E1 mRNA which is declined to about 20% of the original level after 1 day of culture and is unmeasurable after 3 days (5).

Examination of cytochrome P450 phosphorylation was carried out as previously described in detail (6). In brief, isolated rat hepatocytes were suspended in phosphate-free buffer, and subsequently transferred to 60 mm culture dishes for 30 min preincubation at 37^{0} with 100 µCi [^{32}P]-phosphate (Amersham)/1.5 ml and, where indicated, ethanol 75 mM. Cells were then challenged with indicated concentrations of adrenalin or insulin for additional 30 min before termination with ice-cold TES-buffer as described (6). Microsomes were prepared as previously described (5) and immunoprecipitated with anti-CYP2E1 or anti-CYP2B1-IgG. The resulting precipitates were washed and subjected to SDS-PAGE, with subsequent autoradiography of dried gels. Incorporation of [^{32}P]-phosphate into cytochrome P450 was analyzed by intensity-scanning of autoradiograms as described elsewhere (6).

The same preparations of hepatocytes were also cultured under serum free conditions for two days, essentially as described by Eliasson et al. (5). The levels of cytochromes CYP2E1 and CYP2B1 were examined by Western blotting of the microsomal fractions (9, 10).

Para-nitrophenol hydroxylase activities were measured on microsomal preparations from the hepatocytes, essentially as described by Koop (11).

RESULTS AND DISCUSSION

Previous experiments in isolated hepatolytes have shown that glucagon causes increased phosphorylation of CYP2E1 and CYP2B1 at a single site, Ser^{129} (6) and Ser^{128} (12), respectively. Stimulation of hepatocytes, isolated from starved and acetone treated rats by adrenalin, caused increased phosphorylation of both CYP2E1 and CYP2B1 at a half maximal effect of 100 nM (Fig 1). The dose response curves were similar, although

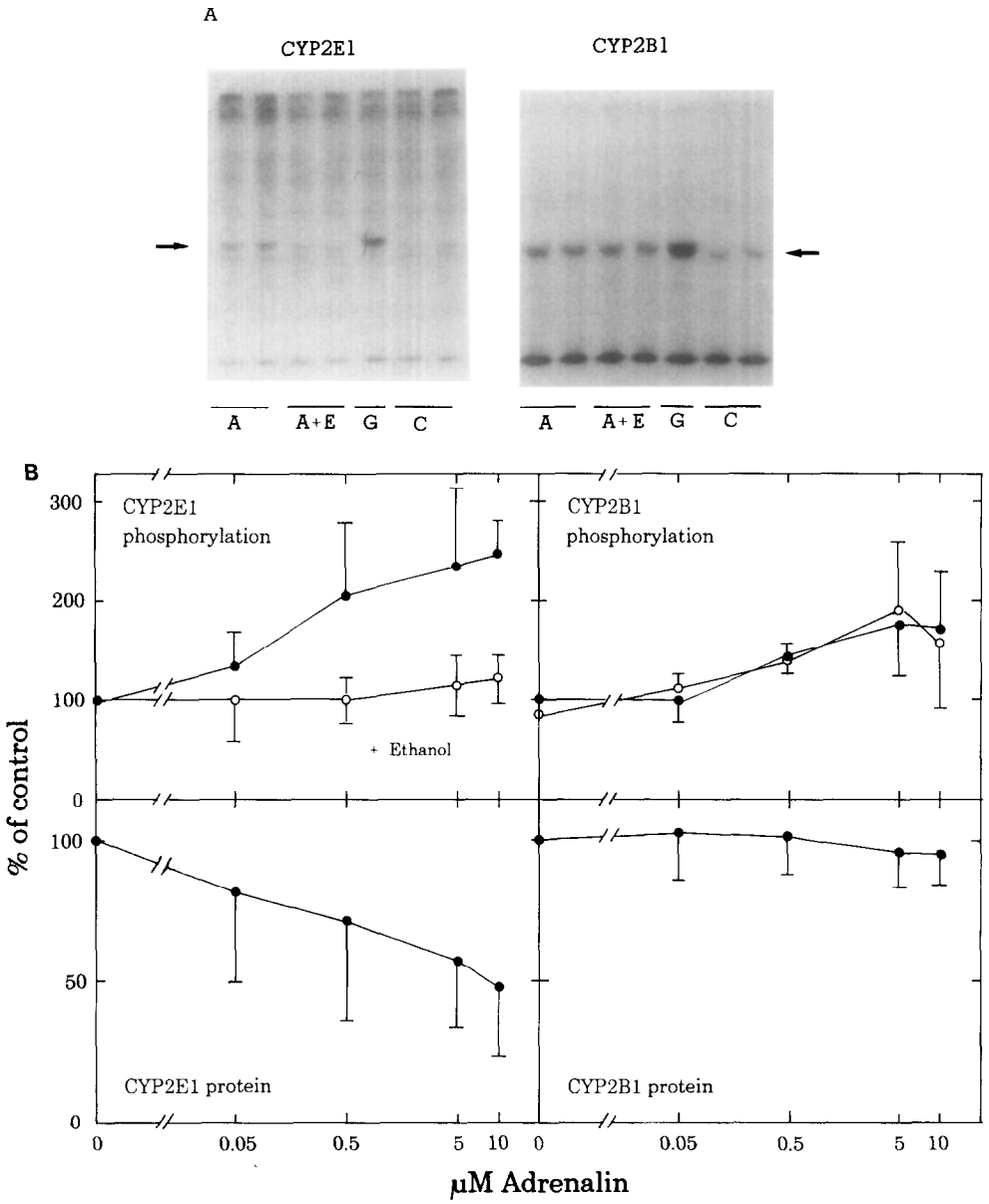

Fig.1. Effect of adrenalin and ethanol on phosphorylation and degradation of CYP2E1 and CYP2B1 in isolated hepatocytes from rats starved and treated with acetone.

A. Autoradiogram of SDS-gel electrophoretic analysis of CYP2E1 (left) and CYP2B1 (right) immunoprecipitates after stimulation of the hepatocytes with adrenalin (10^{-5} M, A), adrenalin and ethanol (10^{-5} M & 75 mM, respectively, A+E), Glucagon ($5x10^{-7}$ M, G) or treated with vehicle (C). Arrow indicates position of CYP2E1 and CYP2B1, respectively. B. Phosphorylation and degradation of CYP2E1 (left) and CYP2B1 (right) in the presence (O---O) or in the absence (●---●) of 75 mM ethanol. The extent of phosphorylation was evaluated after adrenalin treatment for 30 min, whereas degradation was investigated after 48 hours of culture. The amount of CYP2E1 and CYP2B1 was determined by Western blot analysis of isolated microsomes and are expressed in relation to the level present in cell cultures treated with vehicle alone. The data shown are mean ± SEM of four different experiments.

the increase of phosphorylation was higher with CYP2E1. As seen from Fig 1B, the degradation rate of CYP2E1 was increased by adrenalin at the same concentrations that caused phosphorylation. The correlation coefficient between the two adrenalin stimulated events was 0.93. By contrast, adrenalin did not significantly influence the rate of CYP2B1 degradation in the hepatocytes (Fig 1B). Ethanol completely protected CYP2E1 from adrenalin-dependent phosphorylation and degradation, but did not influence phosphorylation or degradation of CYP2B1 (Fig 1, Table I).

Para-nitrophenol hydroxylase has previously been shown to provide a specific catalytic reaction for CYP2E1 (11). In order to investigate whether the form of CYP2E1 stabilized by ethanol, was catalytically active, microsomes were prepared from variously treated hepatocytes and incubated with *para*-nitrophenol. As is evident from Table I, the rate of *para*-nitrophenol hydroxylase activity was about 30-fold faster in hepatocytes cultured in the presence of ethanol (25 mM). This difference was larger than expected from the 8-fold higher levels of CYP2E1 in hepatocytes treated with ethanol, and might indicate the presence of apo-forms of CYP2E1 in the control hepatocytes (see below). Thus, it is evident that ethanol does stabilize the functional form of CYP2E1. These results are compatible with the previously proposed mechansim for CYP2E1 breakdown, involving an initial loss of heme (6).

Table I. *Para*-nitrophenol hydroxylase activity and relative amount of CYP2E1 and CYP2B1, as determined by Western-blotting, in microsomes isolated from hepatocytes cultured for 48 hours

Treatment	Hydroxylase activity nmol/mg, min	CYP2E1 (%)	CYP2B1 (%)
None	0.03±0.02	100	100
Adrenalin, 5 μM	n.d.	68±8	96±5
Ethanol, 25 mM	0.96±0.07	832±156	100±4
Adrenalin, 5 μM + ethanol, 25 mM	1.15±0.35	844±157	99±2

Hepatocytes were isolated from rats starved and treated with acetone as described under Methods. The results represent mean values ± SEM of three different experiments performed in triplicate. The amount of CYP protein is related to the concentration in cultures treated with vehicle only. n.d., not determined.

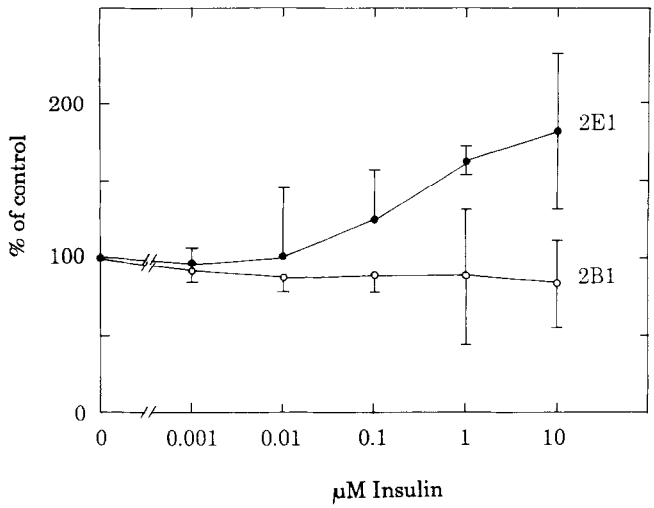

Fig. 2. Effect of insulin on the rate of degradation of cytochrome CYP2E1 (●---●) and CYP2B1 (O---O) in isolated rat hepatocytes.
The cells were incubated with indicated amounts of insulin for 48 hours and the amount of CYP2E1 and CYP2B1 was subsequently determined by Western-blot analysis of isolated microsomes. The data shown are mean ±SEM of three different experiments.

Stimulation of the hepatocyte cultures with insulin, caused stabilization of CYP2E1, but did not affect the rate of CYP2B1 degradation (Fig 2). The half maximal concentration of insulin required was quite high (250 nM) and no significant change in the extent of CYP2E1 phosphorylation could be seen (data not shown).

The results of the present investigation show that the rate of degradation of CYP2E1 is significantly influenced by adrenalin and insulin, whereas CYP2B1 is not affected by these hormones. This indicates separate control mechanisms for their degradation. This is in agreement with previous findings *in vivo*. CYP2E1 is degraded with half lives of 7 h and 37 h, respectively (7). Acetone has the capability to protect the protein from the fast turnover (7). By contrast, induced CYP2B1 is degraded only at a slower rate with a half life of 37 h (13). Further differences *in vivo* concerns the fact that CYP2B1 dependent catalytic activity and protein decline concomitant after induction with acetone, whereas CYP2E1 dependent activities down regulate with a shorter half life than the corresponding CYP2E1 protein, indicating the formation of apo-pools of the enzyme (14). Thus, taken together these data indicate

the existence of at least two principally different mechanisms for cytochrome P450 degradation, one hormone and substrate regulated fast mechanism and one substrate and hormone insensitive mechanism where the protein is degraded in the lysosomes (14, 15).

ACKNOWLEDGMENTS

We are indebted to Mrs Åsa Johansson for excellent technical assistance. This work was supported by grants from Lars Hierttas minne, Magn Bergvalls Stiftelse, Svenska Läkarsällskapet and from the Swedish Medical Research Council.

REFERENCES

1. Rangarajan, P. N. and Padmanaban, G. (1989) *Proc. Natl. Acad. Sci (USA)* 86, 3963-3967.
2. Sogawa, K., Fujisawa-Sehara, A., Yamane, M., and Fujii-Kuriyama, Y. (1986) *Proc. Natl. Acad. Sci. USA.* 83, 8044-8048.
3. Song, B-J., Matsunga, T., Hardwick, J.P., Park, S.S., Veech, R.L., Yang, C.S., Gelboin H.V. and Gonzalez, F.J. (1987) *Mol. Endocrinol.* 1, 542-547.
4. Kim, S.G., States, J.C. and Novak, R.F. (1990) in *Drug Metabolizing Enzymes: Genetics, Regulation and Toxicology* (Ingelman-Sundberg, M., Gustafsson, J.-Å. and Orrenius, S, Eds.) p. 73.
5. Eliasson, E., Johansson I. and Ingelman-Sundberg, M. (1988) *Biochem. Biophys. Res. Commun.* 150, 436-443.
6. Eliasson, E., Johansson, I. and Ingelman-Sundberg, M. (1990) *Proc. Natl. Acad. Sci. (USA)* 87, 3225-3229.
7. Song, B-J., Veech, R.L., Park, S.S., Gelboin, H.V. and Gonzalez, F.J. (1989) *J. Biol. Chem.* 264, 3568-3572.
8. Watkins, P.B., Wrighton, S.A., Schuetz, E.G., Maurel, P. and Guzelian, P.S. (1986) *J. Biol. Chem.*, 261., 6264-6271.
9. Johansson, I., Ekström, G., Scholte, B., Puzycki, D., Jörnvall H. and Ingelman-Sundberg, M. (1988) *Biochemistry* 27, 1925-1934.
10. Johansson, I. and Ingelman-Sundberg, M. (1988) *Cancer Res.* 48, 5387-5390.
11. Koop, D. (1986) *Mol. Pharmacol.*, 29, 399-404.
12. Pyerin, W. and Taniguchi, H. (1989) *EMBO J* 8, 3003-3010.
13. Shiraki, H. and Guengerich, F.P. (1984) *Arch. Biochem. Biophys.* 235, 86-95.
14. Ronis, M.J.J. and Ingelman-Sundberg, M. (1989) *Xenobiotica* 19, 1161-1167.
15. Masaki, R., Yamamoto, A. and Tashiro, Y. (1987) *J. Cell. Biol.* 104, 1207-1215.