BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **228,** 110–114 (1996)
ARTICLE NO. 1624

In Vivo Phenobarbital Treatment Increases Protein Binding to a Putative AP-1 Site in the CYP2B2 Promoter

Amy L. Roe,* Robert A. Blouin,*,† and Georgette Howard†,[1]

Graduate Center for Toxicology and †College of Pharmacy, Division of Pharmacology and Experimental Therapeutics, University of Kentucky, Lexington, Kentucky 40536

Received September 19, 1996

Phenobarbital (PB) is a potent inducer of cytochrome P450 enzymes, particularly CYP2B1/2B2. Although the mechanism(s) of PB induction of CYP2B1/2B2 is not fully understood, current research is focusing on the role PB may play in altering the binding of nuclear proteins to critical DNA response elements in the 5′-flanking region of these genes. In this study, rat liver nuclear proteins were analyzed for DNA binding ability using both a general consensus and a CYP2B2 sequence-specific AP-1 oligonucleotide. We demonstrate that *in vivo* PB treatment enhances protein binding activity to the consensus AP-1 oligonucleotide. Likewise, a putative AP-1 site, identified at -1441 in the CYP2B2 5′-flanking region, also formed a sequence specific DNA/protein complex which was enhanced after PB exposure. These data may support a role of AP-1 in the PB induction mechanism of CYP2B1/2B2.

PB induces the activity of a wide variety of enzymes involved in biotransformation of both endobiotics and xenobiotics (1). The induction of cytochrome P450 enzymes is particularly important due to their role in the hydroxylation of steroids, drugs, and carcinogens/mutagens (2).

Current investigations into the mechanism(s) of the major PB-inducible cytochrome P450 enzymes, CYP2B1/2B2, are focusing on the role PB may serve in altering the binding of nuclear proteins to critical DNA response elements which enhance transcriptional initiation of these genes. Ramsden *et al.*, using transgenic mouse strains containing various rat CYP2B2 constructs, have proposed the role of one or more negative regulatory factors in the regulation of CYP2B2 gene expression; PB may function to modify or relieve these repressors (3). A positive glucocorticoid enhancer (GRE), located 1.3 Kb upstream of the transcription initiation site in the CYP2B2 promoter region, has been functionally identified by Jaiswal *et al.* (4). Using transient transfections of FGC4 hepatoma cells, Shaw *et al.* have shown that the anti-progestin/anti-glucocorticoid, RU486, can inhibit PB-dependent induction, implicating involvement of a steroid receptor in the PB induction mechanism (5). Shephard *et al.*, have identified two DNA sequences in the CYP2B2 promoter that bind rat liver nuclear proteins that are enriched or activated by *in vivo* PB treatment (6). Trottier *et al.*, have also identified a phenobarbital response element upstream of the CYP2B2 promoter (7). The objective of our study was to determine whether *in vivo* PB treatment affected protein binding to a putative AP-1 site at-1441 in the CYP2B2 5′-flanking region. In addition, AP-1 binding activity was compared using both an acute and chronic *in vivo* PB dosing regimen.

MATERIALS AND METHODS

Chemicals. Sodium phenobarbital was supplied by Ransdell Co. (Louisville, KY). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. Radioisotopes were obtained from Amersham (Arlington, IL).

[1] To whom correspondence should be addressed at College of Pharmacy, Division of Pharmacology and Experimental Therapeutics, University of Kentucky, Rose St., Lexington, KY 40536-0082. Fax: (606)257-7564.

0006-291X/96 $18.00

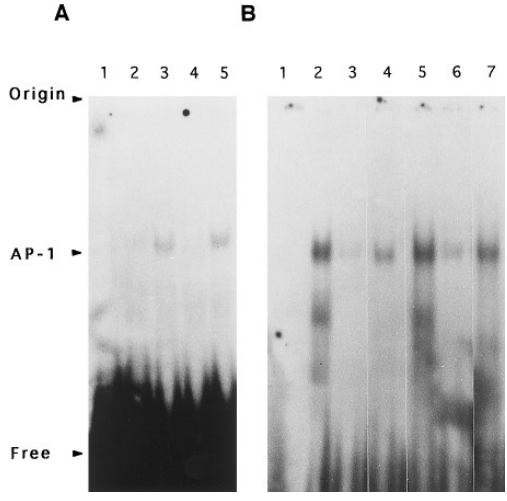

FIG. 1. Electrophoretic mobility shift assay (EMSA) of the AP-1 consensus element. (A) Nuclear extracts were prepared from treatment groups (described in Methods) and incubated with radiolabeled AP-1 consensus as described in methods. Lane 1, free probe without nuclear protein; Lane 2, nuclear extracts (5 Tg) from SDC18h; Lane 3, SDPB18h; Lane 4, SDC5d; Lane 5, SDPB5d. (B) Nuclear extracts (5 Tg) from SDPB18h (lanes 2-4) or SDPB5d (lanes 5-7) were pre-incubated in the presence of either no competitor (lanes 2 and 5) or 100-fold molar excess of unlabeled AP-1 consensus (lanes 3 and 6) or unlabeled SP-1 (lanes 4 and 7). Lane 1 is free probe without nuclear protein.

Animals. Four month old, male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were injected i.p. with either 100 mg/kg PB 18 hours before sacrifice (SDPB18h), or 70 mg/kg PB daily for 4 days and sacrificed on day 5 (SDPB5d). Controls for either injection received equivalent volumes of normal saline (SDC18h, SDC5d).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from rat liver according to Howard *et al.* (8). Protein concentrations were quantitated by the Bradford method (9). HeLa nuclear extracts induced with phorbol 12-myristate 13-acetate (phorbol ester) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

The consensus sequence for AP-1 was purchased from Promega Corp., Madison, WI. A CYP2B2 sequence-specific oligonucleotide was synthesized at the University of Kentucky Macromolecular Structural Analysis Facility (Lexington, KY), and annealed to form a double-stranded 25 bp element (2B2/AP-1). Both oligonucleotides were radiolabeled with [K-^{32}P]dATP using T4 kinase (GIBCO-BRL, Long Island, NY).

EMSAs were carried out as described by Howard *et al.* (8). Competition studies were carried out by preincubation for 20 min. at 220C, in reaction buffer containing 10-to 100-fold molar excess of unlabeled self-competitor oligonucleotide or an unrelated DNA sequence of similar size (SP-1, Promega Corp).

RESULTS

The effects of PB on liver nuclear proteins that may potentially influence CYP2B1/2B2 gene expression were examined after acute (18 hour) or chronic (5 day) *in vivo* exposure to PB. An AP-1 consensus sequence, previously defined for the ability to bind AP-1 protein complex from HeLa nuclear extracts after induction by phorbol esters (10), was tested for nuclear protein binding.

```
                    -1456                                  -1432
CYP2B2/AP-1 .....GTGGATTTNTGACTCATTAGGTTGA.....

AP-1 Consensus              TGACTCA
```

FIG. 2. A comparison of the sequence of the putative AP-1 binding site in the CYP2B2 gene to an AP-1 consensus sequence. The sequence of a segment from the 5′ flanking region of the CYP2B2 gene extending downstream from position -1456 is shown with the putative AP-1 binding site in bold letters. The AP-1 consensus sequence shown is from Bohmann *et al.* (10).

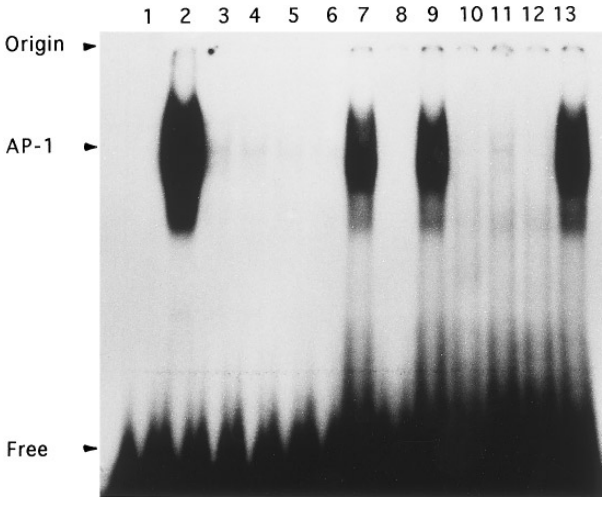

FIG. 3. Cross competition by either the consensus AP-1 or 2B2/AP-1 for phorbol ester-induced HeLa nuclear protein (2.5 Tg) binding. Lanes 1-7 represent labeled consensus AP-1 and competition by 2B2/AP-1 at 0-, 10-, 50-, or 100-fold molar excess. Lanes 8-13 represent labeled 2B2/AP-1 and competition by consensus AP-1 at either 0-, 10-, 50-, or 100-fold molar excess. Lanes 1 and 8, free probe without nuclear protein.

PB treatment of rats did result in a 5-to 7-fold increase in protein binding to this AP-1 consensus sequence (Figure 1). The magnitude of binding did not differ with acute or chronic doses of PB. DNA binding was sequence specific as determined by competition with unlabeled self oligonucleotide but not an unrelated sequence (SP-1, Figure 1).

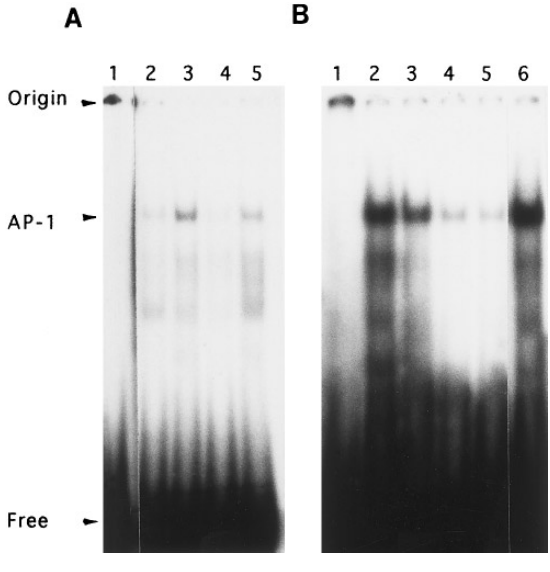

FIG. 4. EMSA analysis of an AP-1 site located within the 5′-flanking region of the CYP2B2 gene (CYP2B2/AP-1). (A) Nuclear extracts (5 Tg) were incubated with radiolabeled 2B2/AP-1 as described in the legend to Figure 1. (B) Titration of nuclear protein binding to the 2B2/AP-1 element. Nuclear extracts were pre-incubated in the presence of 0-, 10-, 50-, and 100-fold molar excess of unlabeled AP-1 (lanes 2-5) or 100-fold molar excess of unlabeled SP-1 (lane 6). Lane 1, free probe without nuclear protein.

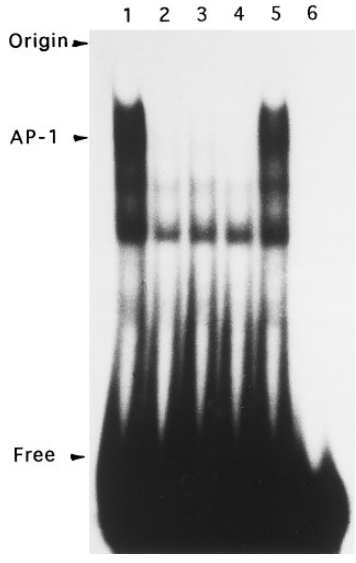

FIG. 5. Cross-competition for liver protein binding to the consensus AP-1 sequence by the CYP2B2 sequence-specific AP-1. Liver nuclear extracts (5 Tg), isolated after 5 days of PB exposure (SDPB5d), were incubated in the presence of 0-, 10-, 50-, and 100-fold molar excess of the 2B2/AP-1 element (Lanes 1-4). Lane 5, 100-fold molar excess of an unrelated SP-1 element; Lane 6, free probe without nuclear protein.

Computer analysis of CYP2B2 gene sequences identified a site identical to the AP-1 consensus sequence (Figure 2) at position-1441 within the 5-flanking region. To characterize the CYP2B2 sequence as an AP-1 binding site, a series of binding studies was performed with phorbol ester-induced HeLa nuclear extracts and liver nuclear extracts from PB-induced rats. Figure 3 demonstrates cross-competition for AP-1 binding by the 2B2/AP-1 sequence where a HeLa protein complex bound by the labeled AP-1 consensus sequence is specifically and effectively competed by an excess of unlabeled 2B2/AP-1 sequence (lanes 1-7). Likewise, the labeled 2B2/AP-1 sequence can form an AP-1-like complex with HeLa nuclear proteins which is also effectively competed by the similar AP-1 consensus sequence (lanes 8-13). Analysis of the CYP2B2/AP-1 sequence in EMSA assays detected liver nuclear protein binding which was consistent with the mobility and PB induction pattern seen with the consensus AP-1 sequence (Figure 4). A similar cross-competition profile to those with phorbol ester-induced HeLa nuclear extracts was seen with liver nuclear extracts from PB-treated rats (Figure 5).

DISCUSSION

In analyzing a region of the CYP2B2 gene spanning from-1402 to +165, Shephard *et al.*, have identified two DNA sequences, located between-183 to-199 and-31 to-72 of the CYP2B2 promoter, that bind rat liver nuclear proteins that are enriched or activated by *in vivo* PB treatment (6). In later studies by this same group, the distal sequence was shown to interact with a member of the octamer transcription family and may be involved in the tissue-specific expression of CYP2B2, whereas the proximal sequence was shown to interact with members of the C/EBP family forming heterodimers with other transcription factors (11). Trottier *et al.*, in transient transfections of primary rat hepatocytes, have identified a phenobarbital response element (PBRE) located between nt-2155 and-2318 of the CYP2B2 gene (7). This large 163 bp fragment, when used in EMSAs, resulted in a complex binding pattern consisting of multiple bound complexes. Upadhya *et al.* and Ram *et al.* have described a minimal PB-

responsive promoter in the CYP2B1/2B2 gene by construction of a minigene consisting of 179nt of the 5′-upstream region (12, 13). The regions-69 to-98nt and-126 to-160nt have been identified as positive and negative regulatory elements. The positive element includes the 'barbie box element'; a highly conserved 17 bp sequence found in a variety of genes that encode barbiturate-inducible proteins, including the CYP2B2 gene (14, 15). Identification of a putative AP-1 site at-1441 in the CYP2B2 gene described in our studies indicates another potential regulatory site worthy of further investigation; however, the precise role of this sequence in mediating the induction of CYP2B1/2B2 in rat liver remains to be delineated.

In summary, this is the first report of an increase in AP-1 binding after *in vivo* exposure to PB. Although others have reported the presence of AP-1-like sequences in the CYP2B2 promoter based on similarity to the AP-1 consensus (16), we are the first to demonstrate and define nuclear protein binding to the putative AP-1 site in the CYP2B2 gene. Furthermore, this increase in binding activity occurs within 18 hours of an acute *in vivo* exposure to PB and persists with chronic treatment for 5 days. These results provide further evidence to support a role for AP-1 transacting factors in the molecular mechanism(s) of PB-dependent induction of cytochrome P450 enzymes and possibly other PB-inducible enzyme systems. Future investigations into the transcriptional nature of the increased AP-1 binding activity and its relationship to other identified elements within the CYP2B2 gene may provide insight into the pleiotropic response of cells to PB.

ACKNOWLEDGMENTS

A.L.R. is supported by NIEHS Training Grant 5 T32 ES07266. Research was supported in part by Sigma Xi Grant-in-Aid of Research Award (A.L.R.) and the University of Kentucky Medical Center. The authors thank Ms. Mary Gayle Engle for photographical assistance.

REFERENCES

1. Waxman, D. J., and Azaroff, L. (1992) *Biochem. J.* **281,** 577–592.
2. Porter, T. D., and Coon, M. J. (1991) *J. Biol. Chem.* **266,** 13469–13472.
3. Ramsden, R., Sommer, K. M., and Omiecinski C. J. (1993) *J. Biol. Chem.* **268,** 21722–21726.
4. Jaiswal, A. K., Haaparanta, T., Luc, P. V., Schembri, J., and Adesnik, M. (1990) *Nucleic Acids Res.* **18,** 4237–4242.
5. Shaw, P. M., Adesnik ,M., Weiss, M. C., and Corcos, L. (1993) *Mol. Pharmacol.* **44,** 775–783.
6. Shephard, E. A., Forrest, L. A., Shervington, A., Fernandez, L. M., Ciaramella, G., and Phillips, I. R. (1994) *DNA Cell Biol.* **13,** 793–804.
7. Trottier, E., Belzil, A., Stoltz, C., and Anderson, A. (1995) *Gene* **158,** 263–268.
8. Howard, G., Keller, P. R., Johnson, T. M., and Meisler, M. H. (1989) *Nucleic Acids Res.* **17,** 8185–8195.
9. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
10. Bohmann, D., Bos, T. J., Admon, A., Nishimura, T., Vogt, P. K., and Tijan, R. (1987) *Science* **238,** 1386–1392.
11. Phillips, I. R., Dell, H., Elia, A., Ciaramella, G., and Shephard, E. A. (1996) Abstracts of papers at the 11th International Symposium on Microsomes and Drug Oxidations, University of California at Los Angeles, July 21–24, Abstract No. L-61, p. 89.
12. Upadhya, P., Venkateswara Rao, M., Venkateswar, V., Rangarajan, P. N., and Padmanaban, G. (1992) *Nucleic Acids Res.* **20,** 557–562.
13. Ram, N., Venkateswara Rao, M., Prabhu, L., Nirodi, C. S., Sultana, S., Vatsala, P. G., and Padmanaban, G. (1995) *Arch. Biochem. Biophys.* **317,** 39–45.
14. He, J. S., and Fulco, A. J. (1991) *J. Biol. Chem.* **266,** 7864–7869.
15. Liang, Q., He, J. S., and Fulco, A. J. (1995) *J. Biol. Chem.* **270,** 4438–4450.
16. Hoffman, M., Mager, W. H., Scholte, B. J., Civil, A., and Planta, R. J. (1992) *Gene Expr.* **2,** 353–363.