Protein Synthesis Inhibitors Exhibit a Nonspecific Effect on Phenobarbital-inducible Cytochrome P450 Gene Expression in Primary Rat Hepatocytes*

(Received for publication, August 26, 1997, and in revised form, November 13, 1997)

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Phenobarbital (PB)1 is well recognized for its pleiotropic effects on mammalian cells, including its ability to transcriptionally activate a variety of genes (1, 2). However, identification of the molecular mechanisms controlling these inductive responses has proven difficult. Certain cytochrome P450s (CYP), including the rat CYP2B1 and CYP2B2 genes, are highly responsive to PB in liver hepatocytes, and provide excellent models for mechanistic studies of the induction process (1, 3). PB and other PB-like inducers do not share obvious structural homology or chemical enantioslectivity (4), as typically associated with classical receptor-mediated gene activation responses. Using a highly differentiated hepatocyte culture system, we previously provided evidence for involvement of distinct intracellular signaling pathways that act in concert to modulate PB induction. Elevation of intracellular cAMP/protein kinase A associated activity by physiological hormones, or other protein kinase A activators, completely repressed induction (5), implying a negative modulatory role for this pathway. Recently, we demonstrated that inhibition of a PP1/PP2A protein phosphatase pathway also effectively suppressed the PB-induction response (6). Thus, the latter pathway may serve as a positive signaling intermediate in the induction process. These results led us to hypothesize that a dephosphorylation event is a required trigger, upstream of PB-mediated transcriptional activation.

In potential conflict with this line of reasoning, results of earlier studies have led some to conclude that another, distinct control mechanism is involved in PB induction. For example, Burger et al. (7) proposed a requirement for de novo protein synthesis in the induction of rat CYP2B gene expression. This conclusion was based on the 46% inhibition of PB-inducible CYP2B1/2 mRNA expression following treatment of primary hepatocytes with 10 μM cycloheximide. In contrast, PB-inducible CYP3A1 gene expression was enhanced 2–19-fold in this study by cycloheximide. These results were interpreted to indicate that PB-mediated intracellular signaling displayed divergence in its mode of induction of the CYP2B versus the CYP3A gene family members. A report by Dogra et al. (8) indicated that in vivo injections of cycloheximide treatment alone activated the levels of PB-inducible CYP2H1 mRNA in chick embryo livers. In fact, these authors demonstrated that coadministration of cycloheximide and PB resulted in a superinduction of the CYP2H1 gene, similar effects to that observed with the dioxin-inducible CYP1A1 gene (9, 10). These findings imply that a labile repressor protein is involved in modulating PB-inducible gene expression in the chicken. Honkakoski et al. (11) used 10 μM cycloheximide treatment of primary mouse hepatocyte cultures and reported that this regimen was ineffective on the Cyp2b10 PB induction status. The latter results suggest that the PB-induction process in the mouse involves a preexisting pathway, and does not require de novo protein synthesis (12).

In the current investigation, we attempted to more fully characterize the potential role of de novo protein synthesis in PB induction. Using a well characterized primary rat hepatocyte culture model, we conducted concentration-effect studies for a broad range of protein synthesis inhibitors, and for an inactive analog, puromycin aminonucleoside. Despite its lack of effect on de novo protein synthesis, the latter agent produced dose-dependent repression of PB induction. Dose-response analyses with the active protein synthesis inhibitors revealed the lack of consistent correlation between extent of protein synthesis inhibition and repression of PB induction. With the exception of puromycin aminonucleoside, all of the protein syn-

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* This work was supported by United States Public Health Service Grant GM32281 (to C. J. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: PB, phenobarbital; CYP, cytochrome P450; SAPK, stress-activated protein kinase; JNK, c-Jun kinase.
thesis inhibitors tested were potent activators of the stress-
activated signal transduction pathway (SAPK/JNK) pathway, 
demonstrating a lack of specificity of these commonly used 
inhibitors. We conclude that, in rat hepatocytes, there is no 
requirement for de novo protein synthesis in the PB induction 
of CYP mRNA expression.

EXPERIMENTAL PROCEDURES

Cell Culture Materials and Chemicals—Unless otherwise stated, all 
cell culture media and Trizol™ (RNA isolation reagent) were obtained 
from Life Technologies Inc. Matrigel™, ITS+ (insulin, transferrin, 
serum-free protein-4 fraction of fetal bovine serum albumin and 
NaCl) and Nu-Sera™ were obtained from Collaborative Biomedical 
Products (Bedford, MA). Leucine-free Dulbecco’s modified Eagle’s medium/F-12 medium 
was purchased from Sigma. Tissue culture-treated plastic flasks (75 cm²) 
were from Falcon (Franklin Lakes, NJ). ³H-Labeled leucine was pur-
chased from NEN Life Science Products. Anisomycin, cycloheximide, 
etetine, puromycin, puromycin aminonucleoside, and dexamethasone 
(9α-fluoro-16a-methyl-11,17a,21-trihydroxy-1,4-pregnadiene-3,20-di-
one) were obtained from Sigma, as were all other unspecified chemicals 
(of the highest grade possible). The phospho-specific SAPK/JNK anti-
body kit was from New England Biolabs (Beverly, MA).

Isolation and Culture of Hepatocytes—Rat hepatocytes were isolated 
by a modification of the two-step collagenase perfusion in situ (13) and 
cultured with a modification (14, 15) of the protocol described previously 
(16).

Gene Induction Treatments—Anisomycin and cycloheximide were 
dissolved in Me₂SO as stock solutions (20 mM) and stored at −20 °C. 
Emetine, puromycin, and puromycin aminonucleoside were dissolved in 
tissue culture-grade water as stock solutions (20 mM) and stored as 
stock solutions (Ecoscint A, National Diagnostics, Atlanta, GA).

RNA Analysis—Total RNA was isolated (17) using Trizol™ as described 
elsewhere (15) from cells pooled from one 75-cm² flask for each 
treatment and analyzed as described previously (15, 18).

Assessment of Protein Synthesis in Primary Hepatocytes—Primary 
hepatocytes were cultured for 48 h. Prior to measurement of protein 
synthesis, cells were washed three times with 95% ethanol and then dried under a 
vacuum. Filter-bound radioactivity was measured by liquid scintillation 
10% trichloroacetic acid 

with different hepatocyte preparations.

Inhibition of de Novo Protein Synthesis in Primary Hepa-
tocytes—Various inhibitors were examined for their ability to 
inhibit de novo protein synthesis in cultures of primary rat hepatocytes. Cells were treated with increasing concentrations of inhibitors for 30–60 min prior to the addition of 
[³H]leucine. Following an additional 60-min incubation, protein synthesis activity was assessed by the measure of label incorporation. 

RESULTS

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[³H]leucine. Following an additional 60-min incubation, protein synthesis activity was assessed by the measure of label incorporation. 

With the exception of the inactive analog of puromycin, 
puromycin aminonucleoside, all the inhibitors effected a concentration-dependent inhibition of de novo protein synthe-
sis (Fig. 1). The relative potency of inhibition is in the order: 
etetine > anisomycin > cycloheximide > puromycin, with the 
last named agent exhibiting only marginal inhibition at a concentra-
tion of 1 µM. In fact, 90–95% inhibition of protein syn-
thesis was achieved only with emetine and anisomycin, at 10 
µM concentrations. Cycloheximide and puromycin exerted only 
80% and 60% inhibition, respectively, at a similar concentra-
tion. Puromycin aminonucleoside was without effect on de novo 
protein synthesis at all concentrations examined.

Inhibition of de Novo Protein Synthesis (Fig. 1) with the 
addition of 1.5 ml of ice-cold cell lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na₃EDTA, 1 mM EGTA, 1% Triton, 2.5 mM 

FIG. 1. Inhibition of de novo protein synthesis in primary rat hepatocytes. Primary hepatocytes were cultured for 72 h under the conditions described under “Experimental Procedures.” Increasing concentrations (µM) of various inhibitors of protein synthesis were added 60 min prior to the incorporation of [³H]leucine for a further 60 min. Total radioactivity was determined in the trichloroacetic acid precipitate as described in “Experimental Procedures” and is expressed as percent activity relative to untreated cells. △△△△, anisomycin; □□□□, cycloheximide; ●●●●, emetine; ○○○○, puromycin; ●○○○, puromycin-aminonucleoside.

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protein synthesis in the PB induction

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Effect of Protein Synthesis Inhibitors on PB Induction—

After establishing the concentration-effect relationships for the various inhibitors and the inactive analog, we next examined the efficacy of these agents as modulators of PB-inducible CYP gene expression. Hepatocytes were treated for 60 min with the same concentration range of inhibitors and then continuously in the absence (control) or presence (PB) of 500 μM phenobarbital for a total of 24 h before RNA harvest. The results of RNA slot-blot hybridization analyses are presented in Fig. 2, A and B.

Anisomycin treatment of primary hepatocytes resulted in only partial inhibition of PB induction of CYP2B and CYP3A1 gene expression. When the induction of CYP2B1 was normalized to ribosomal 18 S RNA (Fig. 2C), only 65% inhibition was effected at 10 μM anisomycin. The identical concentration of
this agent produced >95% inhibition of de novo protein synthesis. In contrast, at a similar concentration, cycloheximide was a more effective inhibitor of PB induction (100% inhibition) than de novo protein synthesis (80% inhibition). Further contrasting results were obtained with puromycin and its inactive analog, puromycin aminonucleoside. Puromycin was markedly more potent than both anisomycin and cycloheximide as an inhibitor of PB-inducible CYP2B1/2 and CYP3A1 gene expression, and yet was least potent with respect to inhibition of de novo protein synthesis. This conclusion was most evident when comparing the 5% inhibition of protein synthesis obtained at 1 μM puromycin versus the approximately 95% inhibition of CYP2B1 induction. Surprisingly, puromycin aminonucleoside also produced a parallel and dramatic concentration-dependent inhibition of PB induction, despite being completely ineffective as a protein synthesis inhibitor. With respect to its potency, puromycin aminonucleoside was less effective than either cycloheximide or puromycin at inhibiting the PB-induction process, but considerably more potent than anisomycin. Cycloheximide and puromycin treatments resulted in only modest alterations of albumin mRNA expression levels.

We also examined the effects of these agents following a shorter time of exposure. Hepatocytes were treated for a total period of 8 h with either 10 μM of anisomycin, cycloheximide, emetine, puromycin, and/or puromycin aminonucleoside. Total RNA was isolated and evaluated by slot-blot RNA hybridization as described under "Experimental Procedures" (panel A). Albumin mRNA levels were included to evaluate the effect of the inhibitors on a liver-selective gene. Ribosomal 18 S RNA hybridization levels were used as normalization standards to demonstrate equal loading of RNA. Autoradiographic data of PB-inducible CYP2B1 mRNA expression was normalized to 18 S ribosomal RNA hybridization data, as for Fig. 2. Normalized signal values are expressed relative to PB-induction response in the absence of inhibitor treatment and are presented as percent CYP2B1 induction (panel B).

**Fig. 3. Effect of a decreased time course of exposure to protein synthesis inhibitors on PB-inducible CYP2B1 mRNA expression.** Primary rat hepatocytes were treated with 10 μM of anisomycin, cycloheximide, emetine, puromycin, and/or puromycin aminonucleoside for 60 min prior to, and then continuously in the absence (C, control) or presence (PB) of 500 μM phenobarbital for a total of 8 h. In addition, hepatocytes were treated with 25 μM of anisomycin, puromycin, and/or puromycin aminonucleoside. Total RNA was isolated and evaluated by slot-blot analysis as described under "Experimental Procedures" (panel A). Albumin mRNA levels were included to evaluate the effect of the inhibitors on the liver-selective gene. Ribosomal 18 S RNA hybridization levels were used as normalization standards to demonstrate equal loading of RNA. Autoradiographic data of PB-inducible CYP2B1 mRNA expression was normalized to 18 S ribosomal RNA hybridization data, as for Fig. 2. Normalized signal values are expressed relative to PB-induction response in the absence of inhibitor treatment and are presented as percent CYP2B1 induction (panel B).

**Effects of Protein Synthesis Inhibitors on the SAPK/JNK Pathway—**After noting the differential effects of protein synthesis inhibitors on PB-mediated CYP gene expression, we questioned the specificity of these agents. In particular, we hypothesized that these substances were effecting other intracellular pathways, independent of their inhibitory properties on de novo protein synthesis. We tested this hypothesis by examining the ability of the inhibitors to stimulate phosphorylation of the SAPK/JNK kinase (p46 and p54 kinases) cascade. Primary hepatocytes were treated with 10 μM concentrations of the protein synthesis inhibitors for time periods ranging from 5 min to 24 h, after which total cell extracts were prepared. The results of Western blot immunoanalyses of differential phosphorylation of the p46 and p54 kinases (SAPK/JNK) between 5 and 60 min are presented in Fig. 4A (panels a, c, and e). These results were normalized to the corresponding levels of immunoreactive p46 and p54 proteins (panels b, d, and f) and are graphically represented in Fig. 4B. Normalized signal values are expressed as relative phosphorylation (arbitrary units) in Fig. 4B.

Cells treated with Me₂SO (control) alone exhibited no observable phosphorylation of this cascade. However, when cells were treated with anisomycin, a rapid and time-dependent increase resulted in phosphorylation of both p46 and p54 SAPK/JNK kinases (Fig. 4B). Concomitant phosphorylation of the c-Jun transcription factor also was observed (data not shown). Longer exposures (2–24 h) resulted in a complete reversion of the stimulation back to control levels (data not shown). We also noted a differential stimulation of the SAPK/JNK cascade with the other inhibitors. Cycloheximide was more effective in stimulating phosphorylation of the cascade than either emetine or puromycin (Fig. 4B). Puromycin exhibited only marginal stimulation after 60 min. However, puromycin aminonucleoside was completely ineffective at stimulating SAPK phosphoryla-
FIG. 4. Time-dependent stimulation of SAPK/JNK phosphorylation by various inhibitors of de novo protein synthesis. Panel A, primary hepatocytes were cultured for 72 h prior to stimulation with 10 μM of protein synthesis inhibitors for a time period of 5–60 min. In addition cells also were treated with epidermal growth factor (EGF) (40 ng/ml) for 5–60 min to assess positive modulation of the mitogen-activated protein kinase (p42/p44) cascade. At each time point shown, cells were lysed, and total cell extracts were prepared as stated under “Experimental Procedures.” Twenty micrograms of total cell extract protein were resolved by SDS-polyacrylamide gel electrophoresis Western analysis. Blots were probed with a phospho-specific antibody directed against phosphorylated SAPK/JNK (a, c, and e), and results were normalized to the corresponding levels of immunoreactive p46 and p54 proteins (b, d, and f). Normalized signal values are expressed as relative phosphorylation (arbitrary units) in panel B.
Effect of Protein Synthesis Inhibitors on PB Induction

In this investigation we used four distinct inhibitors of de novo protein synthesis to more carefully ascertain the role of de novo synthesis on the PB-induction process. These analyses were conducted in a well characterized primary rat hepatocyte model, assessing PB-inducible CYP gene expression as a major end point. Our results demonstrated a potent concentration-dependent inhibition of de novo protein synthesis by these agents and a corresponding but differential inhibition of PB-inducible CYP gene expression. In the results obtained by Burger et al. (7), 10 \mu M cycloheximide treatment produced only 46% inhibition of PB-inducible CYP2B gene expression, while PB induction of CYP2A1 gene expression was actually elevated 2–19-fold. The authors postulated that PB exhibits a distinct divergence in its mode of induction of the CYP2B and CYP3A gene family members. In our system, 8 h of 10 \mu M cycloheximide exposure resulted in nearly complete inhibition of PB induction, at both the CYP2B and CYP3A locus. In addition, all of the additional protein synthesis inhibitors tested resulted in a parallel inhibition of PB-induced CYP2B and CYP3A mRNA expression.

In general, we noted differential effects with these agents between their relative percent inhibition of protein synthesis and PB-inducible gene expression. This was especially clear with anisomycin and puromycin, the most and least potent inhibitors of protein synthesis, respectively, while exhibiting converse effects on the relative degree of inhibition of PB induction. These results, coupled with the finding that 10 \mu M puromycin aminonucleoside, a puromycin analog devoid of any protein synthesis inhibitory activity (19) (data herein), produced largely complete inhibition of the PB-induction process, raises serious issue with the tenet that de novo protein synthesis is a requirement in the PB-mediated signaling process (7, 20).

Our results are consistent with those from Negishi and co-workers (11), who reported no observable effect of cycloheximide treatment on the accumulation of the PB-inducible mouse Cyp2b10 mRNA (21). However, the latter as well as previous investigations of this issue all failed to provide 1) any measures of concentration effect on the induction process, 2) measure of the relative efficacy of their test inhibitors on de novo protein synthesis, or 3) use of any negative analogs. Typically, past conclusions regarding the requirement of de novo protein synthesis for the PB-induction process were made on the basis of a single in vivo injection (20, 22), or single concentrations of an inhibitor in cultures of primary hepatocytes (7).

To assess the specificity of the inhibitors, and whether the inhibitors affected other intracellular signaling processes independent of their action on protein synthesis, we examined the SAPK/JNK cascade. Previous studies established that the SAPK/JNK cascade is potently activated by agents such as anisomycin and puromycin (23, 24). Our data establish, for the first time, the differential phosphorylation of the enzymes involved in this pathway consequent to hepatocyte exposure to protein synthesis inhibitors. Anisomycin was clearly the most potent SAPK/JNK activator. Puromycin, despite being the most effective inhibitor of PB induction, was the least effective modulator of SAPK/JNK phosphorylation. Puromycin aminonucleoside, although highly effective in inhibiting PB induction, was completely ineffective in activating SAPK/JNK phosphorylation. We conclude, therefore, that activation of the SAPK/JNK cascade is not a signaling pathway involved in PB induction. Rather, it appears that inhibitors of protein synthesis exert a nonspecific modulation of PB induction that is quite independent of their ability to disrupt protein synthesis or effect the SAPK/JNK cascade. The exact pathway(s) affected by these agents that modulate the PB-induction process remain to be determined.

One could speculate that if de novo protein synthesis was indeed a critical mode of regulating the PB-induction process, then striking differences would result in the relative DNA-binding patterns with nuclear proteins isolated from control and PB-induced extracts. Trottier et al. (25) examined electrophoretic mobility shift assay complexes and observed that signals from complexes C1 to C3 were similar with nuclear extracts from PB-treated than untreated rat liver. In a recent study (12), establishing the role of a 177-base pair activator region in the 5’ region of the PB-inducible mouse Cyp2b10 gene, Negishi and co-workers detected no differences in protein binding patterns from untreated or PB-treated nuclear extracts with any of the sequences identified as important in PB regulation. We reported similar results using extracts from control and PB-induced nuclei and their interaction with the proximal promoter region of the CYP2B1 and CYP2B2 genes (26).

Our recent studies examining the role of cAMP/protein kinase A activators and PPI/PP2A inhibitors in the PB-signaling process indicate that an upstream phosphatase pathway may participate as an activator of PB induction. The dependence on critical phosphorylation/dephosphorylation events is a well characterized phenomenon regulating enzymatic activities as well as the transcriptional status of many genes (27–30). In summary, we interpret our data to indicate that PB induction of CYP mRNA expression in rat hepatocytes is likely to signal through preexisting phosphatase-regulated factors and therefore does not require a de novo protein synthesis event. The precise pathways involved in this process remain to be identified.

Acknowledgment—We gratefully acknowledge the technical assistance of Diane Wing.

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doi: 10.1074/jbc.273.8.4769

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