BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 152–157 (1996)
ARTICLE NO. 1325

Induction of 5-Aminolevulinate Synthase by Drugs Is Independent of Increased Apocytochrome P450 Synthesis

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To investigate the role of newly synthesized apocytochrome P450 (P450) in the regulation of 5-aminolevulinate synthase (ALAS), we overexpressed P450 in primary cultures of chick embryo hepatocytes and measured the subsequent effects on ALAS mRNA by semiquantitative RT-PCR. Hepatocytes were cotransfected with a vector for expression of P450 cDNAs (CYP3A4 or CYP2H1) and a vector directing the synthesis of a cell surface antibody. Transfected hepatocytes were isolated with hapten-coated magnetic beads at different times after electroporation (4, 8 and 20 h). Overexpression of human CYP3A4 was demonstrated by high levels of the corresponding mRNA and apoprotein as analyzed by RT-PCR and western-blot analysis. Similarly, chicken CYP2H1 was expressed to levels even higher than those induced with phenobarbital. However the level of ALAS mRNA did not change in these cells. Our results demonstrate that the induction of ALAS by drugs is not a direct consequence of increased P450 apoprotein synthesis and heme utilization.

5-Aminolevulinate synthase (ALAS) is the first and rate-limiting enzyme in the heme biosynthetic pathway (1). Under physiological conditions the level of ALAS is low in all tissues but the enzyme is greatly elevated in the liver after administration of a wide variety of drugs such as phenobarbital (PB) (1, 2). The same drugs that induce ALAS also induce the synthesis of several members of the microsomal P450 family (3), the major heme-containing proteins in the liver (4). The associated induction of ALAS and P450 ensures an adequate and apparently coordinated supply of heme for assembly of the induced P450 apoproteins to functional holoproteins.

Heme represses hepatic ALAS by a negative feedback mechanism. It inhibits the transport of cytosolic ALAS into mitochondria (5, 6), decreases mRNA stability (7, 8) and may inhibit gene transcription, at least in rats (9). To explain the increased synthesis of ALAS in response to drugs two alternative hypotheses have been proposed: 1) newly synthesized P450 apoproteins utilize heme, so that preexisting inhibitory heme levels are lowered and ALAS repression is reduced (10) and, 2) drugs play a more direct role and induce ALAS through a still unknown mechanism independent of heme (11-13).

The aim of this study was to determine if an increase in apocytochrome P450, and therefore increased incorporation of heme into these apoproteins, would induce ALAS synthesis. Accordingly, we transfected chick embryo hepatocytes with P450 cDNA (CYP3A4 or CYP2H1) and investigated the effect of P450 overexpression on ALAS. Our results demonstrate that the overexpression of P450 apoprotein in chick embryo hepatocytes does not trigger the induction of ALAS, providing direct evidence against a heme-dependent mechanism of ALAS induction by drugs.

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Abbreviations: ALAS, 5-aminolevulinate synthase; CYP, cytochrome P450; PB, phenobarbital, CHX, cycloheximide; RT, reverse transcription; PCR, polymerase chain reaction; bp, base-pairs.

0006-291X/96 $18.00

MATERIALS AND METHODS

Culture and transfection of chick embryo hepatocytes. Hepatocytes were isolated from 17- day-old chick embryos by *in situ* perfusion and collagenase digestion as previously described (14). The isolated hepatocytes were resuspended in phosphate-buffered saline (PBS) and 5×10^7 cells were transfected by electroporation with 20 μg of exogenous DNA in 0.4 cm gap size cuvettes (Invitrogen). Electroporation was performed with a Gene Pulser II Unit of Bio-Rad set at 960 μF and 350 V. Hepatocytes were transferred to 10-cm dishes containing 10 ml of Williams'E medium supplemented with 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and 5% Nu-Serum (Becton Dickinson).

P450 cDNAs and expression vectors. The cDNA of the PB-inducible CYP2H1 (15), was amplified by PCR from PB-induced chick embryo liver cDNA, using specific primers flanking the coding region (forward 5′-gac act tga cat ctc ttc ctc-3′ and reverse 5′-ctg ggc att gac tat cat t-3′) and Pfu, a high-fidelity thermostable polymerase (Stratagene). The amplified DNA (1572 bp) was cloned into the pCR-Script SK(+) vector (Stratagene) and also was sequenced to confirm the cloning of the specific isoform CYP2H1. Human CYP 3A4 cDNA (2.1 kb) (16) and the chicken CYP2H1 cDNA were subcloned into the mammalian expression vectors pCI containing the immediate-early enhancer / promoter region of CMV (Promega). In transient transfection experiments, chick embryo hepatocytes were co-transfected with the pCI expression vector containing the P450s cDNAs and the vector pHook (1.5/1.0 molar ratio) that directs the expression at the cell surface of a single-chain antibody (sFv) against a specific hapten (17, 18). Hepatocytes expressing the sFv on their surface were isolated from the culture by binding to hapten coated magnetic beads at different times after electroporation (Capture-Tech System, Invitrogen).

Semiquantitative RT-PCR analysis. To overcome detection problems due to the small number of isolated cells after transfection we evaluated the relative changes in mRNA levels by semiquantitative RT-PCR.

RNA purification and cDNA synthesis were performed as described (19), except that contaminating plasmid DNA was removed before cDNA synthesis by incubation with 10 units of DNase I (RNase-free) (Boehringer) and 10 units of the ribonuclease inhibitor RNasin (Promega) in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ for 30 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation. The primers for each cDNA amplification were designed to be specific and selective for the predicted sequences. For human CYP3A4 cDNA (16), the forward and reverse primers, 5′-cct tac aca tac aca ccc ttt gga agt-3′ and 5′-agc tca atg cat gta cag aat ccc cgg tta-3′ respectively, produced a predicted 382 bp fragment between positions 1353 and 1734. For chicken CYP2H1, the primers used for RT-PCR analysis were the same as those described above, and amplified a 1572 bp fragment (15). For chicken housekeeping ALAS (20) the primers were the following: forward (position 452) 5′-agg agg atg tga agg aaa tg-3′, and reverse (position 1036) 5′-caa gag tga aga ggg tgg aa-3′, and produced a 585 bp fragment. In parallel we analyzed chicken β-actin (21) as an internal control for normalization. The forward and reverse primers, 5′- ccc tga acc cca aag cca ac-3′ and 5′-gac tcc ata ccc aag aaa ga-3′ respectively, produced a predicted 487 bp fragment between positions 394 and 880.

Appropriate dilutions were empirically determined for each cDNA to ensure that the resulting PCR products were derived only from the exponential phase of the amplification. Under these conditions, the yield of the PCR product is proportional to the input cDNA (22, 23). cDNA (3 μl) was amplified in a 30 μl final volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 9], 50 μM each deoxynucleotide triphosphate, 1 unit AmpliTaq DNA polymerase (Perkin Elmer) and 0.2 μM of each primer.

PCR was performed with a DNA Thermal Cycler (Perkin Elmer) programmed for an initial denaturation of 4 min at 94°C, followed by 27 cycles of 45 s at 94°C, 45 s at 60°C and 45 s at 72°C, and a final extension of 5 min at 72°C. For quantitative analysis, aliquots (25 μl) of the PCR reaction were subjected to electrophoresis on 1.2% agarose gel and the products visualized by ethidium bromide staining. The gel image was digitalized with a Video-Camera (Gel Print 2000I, BioPhotonics Co.), and the intensity of the bands was quantified with the ImageQuant software package (Molecular Dynamics). The results were plotted on a log-log scale against the corresponding dilution factor of the cDNA. The PCR products were considered in the exponential phase of the amplification when the signal detected was proportional to the corresponding cDNA dilution.

Immunoblot analysis. The overexpression of P450 apoprotein was examined by western blot analysis. 9000×g supernatant and microsomes were prepared from homogenates of cultured cells by differential centrifugation (14). Samples were analyzed on 10% SDS-PAGE and proteins were electroblotted to nitrocellulose membranes (Schleicher & Schuell) using a Pharmacia-LKB semi dry blotter, at 0.8 mA/cm^2 for 1 h at room temperature. Transfer was carried out in 48 mM Tris, 39 mM glycine, 0.004 % SDS and 20 % methanol. The nitrocellulose was blocked with 2% non-fat powdered milk in 0.02 M Tris/NaCl (TBS), pH 7.4, containing 0.5% Tween 20 for 1 h. Expressed human P450 3A4 was detected with rabbit antibody against rat CYP3A1 (24) and horseradish peroxidase-conjugated goat anti-rabbit IgG (ECL western blotting detection, Amersham).

RESULTS AND DISCUSSION

The induction of ALAS by drugs has both fundamental and clinical relevance because ALAS induction is not only responsible for adapting heme synthesis to rapid changes in heme

demand but also because it plays a key role in the genetic disorders leading to the hepatic porphyrias (25). In the present study we have investigated the role of newly synthesized P450 apoprotein in the induction of ALAS by drugs. We achieved an increase in apoprotein by overexpressing different P450 cDNAs in primary cultured hepatocytes, and examined the consequences on ALAS mRNA by RT-PCR analysis.

The expression of a foreign cDNA in primary cultured cells by the presently available techniques is hampered by low transfection efficiencies. Because of this, any effect is diluted by the presence of a high percentage of non-transfected cells. To overcome this problem we used the expression vector pHook (17, 18) for a rapid and selective isolation of transfected

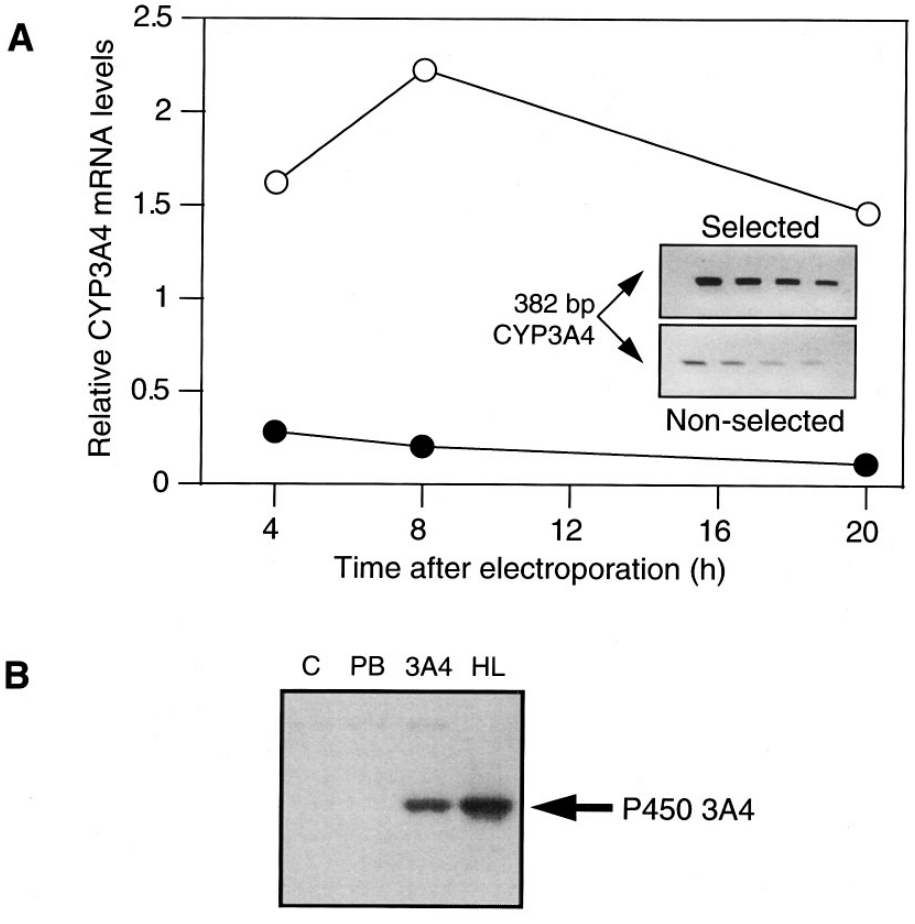

FIG. 1. Overexpression of human CYP3A4 in primary chick embryo hepatocytes. (A) Cells were electroporated with the vectors pCI-CYP3A4 and pHook, and transfected hepatocytes were selected with hapten-coated magnetic beads at 4, 8 and 20 h post-electroporation. CYP3A4 mRNA levels were measured by semiquantitative RT-PCR analysis in selected (○) and non selected cells (●). Values represent arbitrary units of densitometry normalized with the corresponding value for chicken β-actin mRNA. Insert: Representative RT-PCR analysis for CYP3A4 mRNA at 20 h post-electroporation. cDNAs were serially diluted and amplified as described in Materials and Methods and the products were recorded with a video camera after ethidium bromide staining. (B) Microsomes (10 μg) from hepatocytes selected 20 h after electroporation were separated by SDS-PAGE, and CYP3A4 apoprotein levels were analyzed by western-blot using a antibody against rat-CYP3A1. *C*, mock transfected cells; *PB*, mock transfected cells induced 20 h with 1 mM PB; *3A4*, cells transfected with pCI-CYP3A4; *HL*, microsomes from a human liver with high levels of CYP3A4 (HL-26, ref. 26).

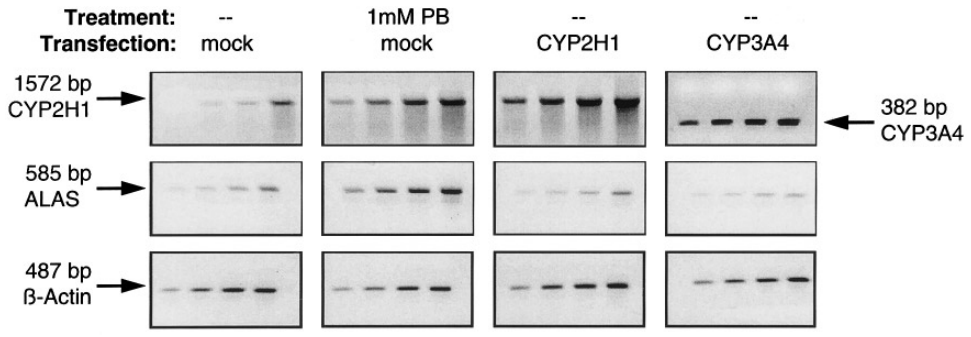

FIG. 2. Representative RT-PCR analysis of chick embryo hepatocytes selected 20 h after transfection. cDNAs from cells transfected with different expression vectors or induced with 1 mM PB were serially diluted and amplified. The PCR products were recorded with a video camera after ethidium bromide staining. Mock transfected cells were electroporated with an amount of pCI-vector and pHook equivalent to that used for transfection of cells overexpressing CYP cDNA. The arrow identifies the cDNA analyzed in each example and the size of the amplified fragment.

cells. Chick embryo hepatocytes transfected with this plasmid expressed a chimeric single chain antibody fused to a transmembrane domain and to a signal peptide that directs it to the plasma membrane (17, 18). As a result, hepatocytes could be isolated with hapten-coated magnetic beads even at short times after electroporation. Cotransfection of hepatocytes with pHook and the expression plasmid pCR3-lacZ encoding β-galactosidase revealed that more than 90% of the selected cells expressed both vectors (data not shown).

We used this experimental approach to overexpress P450 apoprotein in culture of primary hepatocytes. Transfection of cells with the plasmids pCI-CYP3A4 and pHook lead to a rapid increase in CYP3A4 mRNA. Semiquantitative RT-PCR analysis showed that CYP3A4 mRNA levels were 8-11-times higher in selected cells than in non selected cells at several time-points analyzed (Figure 1A). Western-blot analysis revealed that the transfected cDNA was correctly translated into the right size CYP3A4 apoprotein, which accumulated to levels similar to those detected in induced human liver (human liver HL-26 had the highest CYP3A4 apoprotein levels and associated activity (midazolam hydroxylation) out of a group of 27 livers studied (26)) (Figure 1B). The polyclonal antibody against rat CYP3A1 (24) did not detect any other major protein in control or PB-induced cells. Therefore, with this experimental approach, we were able to overexpress a P450 in primary liver cells to high levels and with a similar time-course as that observed after PB induction.

In a second set of experiments we investigated the effect of a homologous (CYP2H1) or heterologous (CYP3A4) P450 overexpression on ALAS mRNA levels. A representative RT-PCR analysis of samples collected 20 h after electroporation and/or PB-induction is shown in Figure 2. The level of CYP2H1 mRNA in transfected cells was approximately two-times higher than in cells induced with 1 mM PB, and 20-fold higher than in the controls (Figure 2 and 3A). However, the level of ALAS mRNA was not affected by this overexpression, whereas PB induced ALAS mRNA 5-fold (Figure 2 and 3A). Similar results were obtained in hepatocytes expressing high levels of human CYP3A4.

We also measured relative changes in ALAS mRNA at shorter times after transfection to exclude the possibility of a rapid and transitory induction of ALAS. The induction of CYP2H1 mRNA was moderate but the levels of ALAS were already substantially induced at 8 h after PB (3-fold) (Figure 3B). In contrast, the expression level of CYP2H1 mRNA in pCI-CYP2H1 transfected cells was 22-fold higher than in the controls, but ALAS mRNA did not show a proportional increase. ALAS mRNA levels were about 1.6-1.7-times higher than the controls in pCI-CYP2H1 transfected cells, at both 4 h (data not shown) and 8 h after electroporation

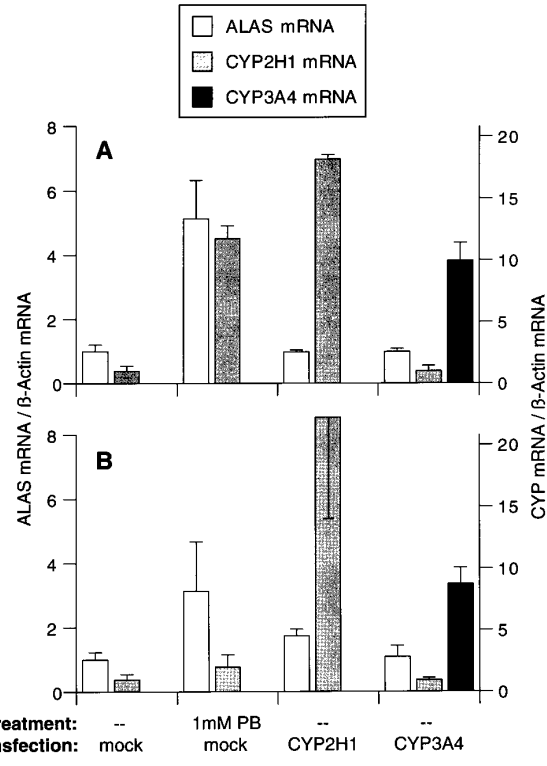

FIG. 3. Relative mRNA levels of ALAS, CYP2H1 and CYP3A4 in chick embryo hepatocytes overexpressing P450 or induced with PB. Cells were selected at 20 h (A) or 8 h (B) after transfection and the relative mRNA levels of ALAS, CYP2H1 and CYP3A4 were estimated by semiquantitative RT-PCR analysis. The values for ALAS and CYP2H1 mRNA are expressed relative to their respective values in control mock transfected cells (represented as 1.0), and the values for CYP3A4 mRNA relative to the levels detected in non-selected cells from the same experiment. Data represent average ± range of 2–4 independent experiments.

(Fig 3B), but this small increase was not observed in cells overexpressing CYP3A4 from the same cultures (Fig 3B).

Our results therefore demonstrate that the overexpression of P450s in chick embryo hepatocytes to levels similar to those induced by PB does not trigger a substantial ALAS induction, suggesting that most of the increased synthesis of this enzyme after PB is not a direct consequence of increased P450 apoprotein and heme utilization, and that PB may play a more direct role in ALAS induction.

Previous studies with the protein synthesis inhibitor cycloheximide (CHX) are in agreement with our conclusion (11-13, 27). Treatment of chick embryo liver or cultured hepatocytes with CHX showed that the PB-induced increase in ALAS transcription was not inhibited but, in fact, was markedly stimulated (13). The data suggested that the induction of ALAS gene transcription is independent of the synthesis of P450 apoprotein, and moreover, that a labile repressor protein may be involved. However, the use of CHX to block the synthesis of P450 apoprotein and hence heme utilization, has several drawbacks. The simultaneous treatment with CHX and PB caused also superinduction of CYP2H1/2 expression, i.e. CYP2H1 gene transcription increased about 45-fold (13). On the other hand, the inhibition of protein synthesis by CHX is not total and is transitory. Therefore, under experimental conditions of P450 superinduction together with 10-15% residual protein synthesis a still significant increase in

P450 apoprotein can not be ruled out. Moreover, in contrast to this superinduction of ALAS in chick embryo liver (13), CHX inhibits the induction of ALAS by PB in rat hepatocytes (33). In addition, the observation of ALAS induction by PB in the absence of protein synthesis is confounded by complex additional effects of CHX and should be confirmed by more direct experimental approaches.

In the present study we have demonstrated that ALAS is not induced by increased concentrations of homologous or heterologous P450 apoprotein. Our results therefore support the general conclusion that drug induction of ALAS is due to a direct effect of the inducing compound and not a consequence of the increased synthesis and accumulation of cytochrome P450.

ACKNOWLEDGMENTS

We thank Doreen Marek for her expert technical assistance in the cloning and sequencing of CYP2H1 cDNA. This study was supported by a Research Training Fellowship of the European Community (Biomedical and Health Programme) and the Swiss National Science Foundation.

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