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Expression of 5-aminolaevulinate synthase and cytochrome *P*-450 mRNAs in chicken embryo hepatocytes *in vivo* and in culture

Effect of porphyrinogenic drugs and haem

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To examine current models for the co-ordinate regulation of 5-aminolaevulinate (ALA) synthase and cytochrome *P*-450 we have determined the effect of drugs, inhibitors of haem biosynthesis, haem and cycloheximide on the steady-state expression of mRNAs for ALA synthase and a phenobarbital-inducible cytochrome *P*-450 (PB_1 *P*-450), in chick embryo hepatocytes *in vivo* and in primary culture. We found that the mRNAs for ALA synthase and PB_1 *P*-450 were rapidly and simultaneously induced by the porphyrinogenic drugs glutethimide and 2-propyl-2-isopropylacetamide. Inhibitors of haem biosynthesis when administered alone had a small effect on ALA synthase mRNA induction, but in combination with the drugs synergistically increased induction of both ALA synthase mRNA and enzyme activity. However, there were concentrations of inhibitors that increased induction of enzyme activity without increasing mRNA induction. Haem suppressed ALA synthase mRNA induction by drugs by only 50%, whereas induction of ALA synthase enzyme activity was completely suppressed. This suppression of ALA synthase mRNA by haem was blocked by cycloheximide treatment which did not block the induction of ALA synthase mRNA by drugs. In fact, cycloheximide synergistically increased the drug induction of ALA synthase mRNA, suggesting the presence of a labile protein factor which may interact with a haem-responsive element of the ALA synthase gene. Cycloheximide treatment alone did not significantly affect ALA synthase mRNA expression, but induced PB_1 *P*-450 mRNA to a similar extent to that caused by porphyrinogenic drugs, suggesting the presence of a labile repressor which modulates PB_1 *P*-450 gene expression. Basal and drug-inducible PB_1 *P*-450 mRNA levels were unaffected by haem or by inhibitors of haem biosynthesis, indicating that the PB_1 *P*-450 gene is not regulated by haem in chick embryo hepatocytes. Our results indicate that drugs simultaneously induce ALA synthase and PB_1 *P*-450 mRNA expression, and that ALA synthase activity is regulated by haem principally at a post-transcriptional site rather than at the transcriptional level.

INTRODUCTION

5-Aminolaevulinate (ALA) synthase (EC 2.3.1.37) is the first and rate-limiting enzyme in the haem biosynthetic pathway in the liver (reviewed in [1,2]). There is both direct and indirect evidence that haem can feedback regulate this enzyme [1,2]. Phenobarbital (PB) and other porphyrinogenic drugs, which can stimulate haem biosynthesis and induce porphyria, also induce ALA synthase activity [1,2]. These same compounds also induce a sub-family of cytochrome *P*-450 isoenzymes in the liver [3], recently classified as the cytochrome *P*-450 IIB sub-family [4]. Since most of the haem synthesized in the liver is incorporated into cytochrome *P*-450s, many investigations of ALA synthase expression have focused on the relationship between drug-mediated induction of ALA synthase and the cytochrome *P*-450s [1,2]. The mechanism of induction of ALA synthase and cytochrome *P*-450s by PB and similar drugs is not known. There is presently no evidence for a receptor-mediated induction pathway analogous to that of the 3-methylcholanthrene-inducible cytochrome *P*-450 I family [3–5].

Both direct and indirect mechanisms for the induction of ALA synthase by drugs have been proposed (reviewed in [6]). The current model, as recently reviewed by May and co-workers [1], proposes that regulation of ALA synthase expression is controlled solely by haem. According to this model, haem acts as a negative regulator at the transcriptional level, and the induction of ALA synthase by porphyrinogenic drugs occurs indirectly as a result of the direct induction of cytochrome *P*-450s by drugs. The depletion of haem which occurs upon incorporation of haem into newly synthesized cytochrome *P*-450 apoprotein to form the holo-enzyme is postulated to lead to a derepression of ALA synthase gene expression [1,2]. We have investigated the mechanism of ALA synthase and cytochrome *P*-450 induction by quantification of steady-state mRNA levels for ALA synthase and a PB-inducible cytochrome *P*-450, which we designate as PB_1 *P*-450

Abbreviations used: ALA, 5-aminolaevulinate; DDC, 3,5-dicarbethoxy-1,4-dihydrocollidine; PB, phenobarbital; PIA, 2-propyl-2-isopropylacetamide; PBS, phosphate-buffered saline.

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in chick embryo hepatocytes *in vivo* and in primary culture using a solution hybridization assay. [The sequence of the major PB-inducible form of cytochrome *P*-450 in chick-embryo liver has been tentatively assigned to the IIC sub-family (*P*-450 IIC10) according to the recently proposed nomenclature [4]. However, its true evolutionary relationship with other cytochrome *P*-450s is presently not clear, since PB_1 *P*-450 is the only avian sequence published to date and shares only a 45–54% amino acid sequence similarity with the published sequence of mammalian PB-inducible *P*-450s.] This assay has enabled us to study the early events in the induction of ALA synthase and PB_1 *P*-450 expression by porphyrinogenic drugs. Our results support a model for regulation of ALA synthase mRNA expression in which porphyrinogenic drugs directly induce mRNA expression and haem acts as a modifier of mRNA induction by drugs. We also propose a new mechanism for the induction of a PB-inducible cytochrome *P*-450 by drugs.

MATERIALS AND METHODS

Chicken embryos

Fertile White Leghorn chicken eggs were obtained from Truslow Farms, Inc., MD, U.S.A. Eggs were incubated at 37.5 °C and 85% humidity in a Petersime model 1 incubator which rotated the eggs once every 2 h. Experiments *in vivo* were performed on 14-day embryos. Glutethimide (Sigma, St. Louis, MO, U.S.A.), 2-propyl-2-isopropylacetamide (PIA, a gift from Hoffman–LaRoche, Nutley, NJ, U.S.A.), and 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC, Kodak, Rochester, NY, U.S.A.; recrystallized) were administered alone or in combination in 10 μl of acetone, and desferrioxamine mesylate (Ciba–Geigy, West Caldwell, NJ, U.S.A.) was administered in 100 μl of water. Chemicals were pipetted onto the inner-shell membrane as previously described [7]. Following treatment, each embryo was opened ventrally, perfused through the heart with phosphate-buffered saline (PBS)/EDTA (136 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4, 10 mM-EDTA, pH 7.4), and the liver was removed and rinsed in PBS/EDTA. A piece of each liver was frozen at −70 °C for subsequent RNA isolation. The remainder of the liver was used for ALA synthase and *P*-450 determinations.

Hepatocyte cultures

Primary cultures of chick embryo hepatocytes were prepared from 15-day-old embryos as previously described [8]. Liver cells were cultured on 10 cm diameter dishes in serum-free William's E medium containing insulin (0.2 μM), dexamethasone (0.8 μM) and 3,3',5-triiodothyronine (2 μM), at a cell concentration of $(8–12) \times 10^6$ cells/dish. After 20 h in culture, the medium was changed to serum-free William's E medium containing dexamethasone and tri-iodothyronine, but not insulin, and cells were incubated for an additional 20 h prior to drug treatment. Cultures were approximately 70–80% confluent at the time of treatment. Cultures were treated with drugs in 50–100 μl of solvent. Desferrioxamine, cycloheximide (Sigma) and succinylacetone (4,6-dioxoheptanoic acid, U.S. Biochemical, Cleveland, OH, U.S.A.) were dissolved in water and PIA was dissolved in 50% aqueous ethanol. For experiments involving measurement of ALA synthase activity and cytochrome *P*-450 concentrations, the culture dishes were placed on ice, the medium was removed, and the cells were washed once with ice-cold PBS/EDTA. Cells were scraped from two to three 10 cm diameter dishes and pooled. After removal of aliquots of each cell suspension for ALA synthase and cytochrome *P*-450 determinations, cells were pelleted by centrifugation at 1000 *g* for 15 min and 4 °C, and frozen at −70 °C for subsequent RNA isolation. For experiments involving mRNA measurements only, cells were harvested by adding 4.4 ml of guanidine isothiocyanate solution [4 M-guanidine isothiocyanate, 10 mM-Tris, 7% (v/v) 2-mercaptoethanol, 2% Sarkosyl, pH 7.4] directly to washed culture dishes and RNA was isolated as described below.

Measurements of ALA synthase activity and cytochrome *P*-450

For ALA synthase determinations on embryo liver, 10% (w/v) homogenates were prepared in ALA synthase assay buffer (35 mM-Tris, 30 mM-sodium phosphate, 8 mM-$MgCl_2$, 5 mM-EDTA, 15 mM-sodium citrate, 10 mM-sodium laevulinate, 0.5 mM-pyridoxal-5'-phosphate, pH 7.4), and for cytochrome *P*-450 determinations, microsomes were isolated from pooled homogenates as previously described [9]. Cultured cells were homogenized in ALA synthase assay buffer. ALA synthase activity was assayed as previously described [10], except that the reaction was initiated by addition of 50 mM-neutralized glycine. Cytochrome *P*-450 was assayed in the embryo-liver microsomes or in detergent-solubilized supernatants from cultured cell homogenates as previously described [9].

RNA isolation

Total RNA was isolated using modifications of previously described protocols [11,12]. Each frozen liver or cell pellet was overlaid with 4.4 ml of guanidine isothiocyanate solution and homogenized by drawing the mixture 10 times through an 18 gauge needle. The homogenate was layered onto 10 ml of CsCl solution (1 g of CsCl/ml in 10 mM-EDTA, pH 7.4, specific gravity 1.76 g/ml) and centrifuged at 55000 $g_{av.}$ (20000 rev./min in Sorvall AH-629 rotor) at 20 °C for 20 h. Following centrifugation the RNA was rinsed with 4 ml of 70% ethanol and centrifuged at 4000 $g_{av.}$ (5000 rev./min) for 30 min at 20 °C. The RNA was redissolved by heating the pellet in 0.25–0.5 ml of water for 5 min at 60 °C. RNA was stored at −70 °C. The final RNA concentration was determined spectrophotometrically assuming $\Delta A_{260-320} = 7330\ M^{-1}\ cm^{-1}$. This procedure yielded RNA that was not detectably degraded (as determined on denaturing agarose gels) and had < 1% DNA or protein contamination.

Preparation of synthetic oligonucleotide probes

The nucleotide sequences of a chicken-liver-specific ALA synthase mRNA and a PB-inducible chicken liver cytochrome *P*-450 mRNA have recently been determined from cDNA libraries [13,14]. Oligomeric cDNA probes specific for each of these sequences were synthesized on an automated DNA synthesizer (Beckman System One) and were subsequently purified by electrophoresis on 20% polyacrylamide/7 M-urea gels followed by chromatography on a NENsorb 20 nucleic acid purification

cartridge (New England Nuclear, Boston, MA, U.S.A.). The ALA synthase probe is a 24 nucleotide cDNA with the sequence $^{5'}$GTAGATCTCACAACCTGGCAG-CAT$^{3'}$, which is complementary to nucleotides 793–816 of the cDNA-derived coding sequence [13], and which has been used previously to quantify ALA synthase mRNA in cultured chick-embryo hepatocytes by solution hybridization [15]. The cytochrome *P*-450 probe is a 24 nucleotide cDNA with the sequence $^{5'}$GTTATTTTC-TTCCAGCATCTCAAT$^{3'}$, which is complementary to nucleotides 640–663 of the cDNA-derived coding sequence [14]. Two putative cytochrome *P*-450 mRNAs with reported sizes of 3.5 kb and 2.5 kb, were originally cloned by Hobbs *et al.* [14] from a cDNA library from ALA-induced chick embryo liver. Our probe is complementary to a region of the 3.5 kb mRNA (PB_1 *P*-450) which was reported not to cross-hybridize to the 2.5 kb mRNA using cDNA probes for this region [14,16]. We have confirmed by Northern-blot analysis that each probe hybridizes to a single band of appropriate size using liver poly A^+ mRNA from glutethimide-induced chick embryos (results not shown). For solution hybridization analysis, each cDNA probe was 5′ end-labelled using [γ-^{32}P]ATP (New England Nuclear, 3000 Ci/mmol) and T4 polynucleotide kinase (Bethesda Research Labs., Gaithersburg, MD, U.S.A.) as previously described [17], and re-purified by NENsorb 20 chromatography. Specific activities for each probe were approx. 5000 c.p.m./fmol or approx. 6×10^8 c.p.m./μg.

Solution hybridization

Solution hybridization of 5′ end-labelled cDNA probes to RNA was performed using modifications of the methods described by Omiecinski *et al.* [17]. Briefly, 5–10 μg of total cellular RNA was hybridized with 15–25 fmol of labelled cDNA probe (approx. 10^5 c.p.m.) in 100 μl of hybridization buffer (0.75 M-NaCl, 0.2% sodium dodecyl sulphate, 4 mM-EDTA/20 mM-Tris, pH 7.5) for 18 h at 55 °C. To this reaction mixture was added 8 units of S1 nuclease (Bethesda Research Labs.) and 300 μl of S1 buffer (0.75 M-NaCl, 2.8 mM-$ZnSO_4$, 70 mM-sodium acetate, 100 μg of sheared salmon-testes DNA, pH 4.5) and the non-hybridized nucleic acids were digested for 1 h at 37 °C. Nucleic acid hybrids were precipitated by addition of 100 μl of 50% (w/v) trichloroacetic acid and incubation at 4 °C for 1 h, and then collected onto individual 7 mm diameter glass filters. The filters were rinsed with 3% (w/v) trichloroacetic acid/1% (w/v) NaH_2PO_4 followed by 95% aqueous ethanol, and radioactivity was quantified by liquid scintillation in Aquasol 2 scintillation fluid (New England Nuclear) using a Searle Mark II scintillation counter.

Four replicate hybridizations were run for each RNA sample and the values were averaged. The S.D. of sample replicates was typically 5–10% of the mean. For each hybridization assay, four control groups were also run in quadruplicate: background counts (filters alone); S1

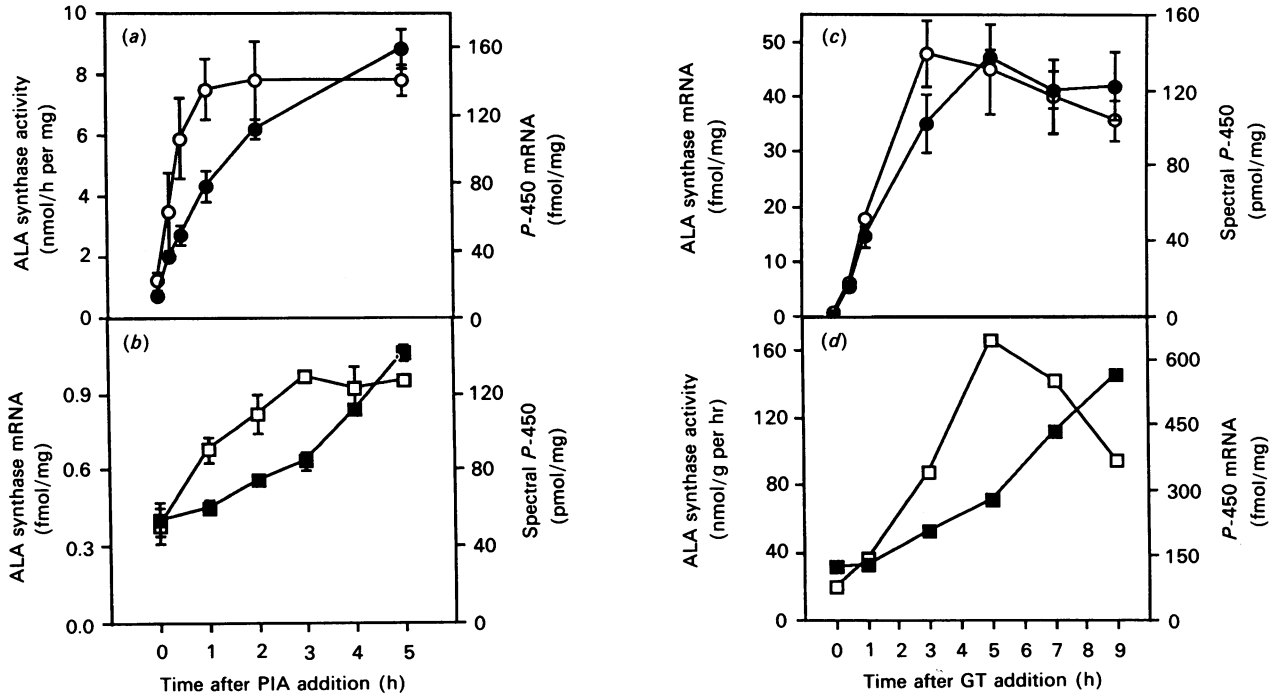

Fig. 1. Time-course of induction of ALA synthase and cytochrome *P*-450 in chick embryo hepatocytes *in vivo* and in culture

Cultured hepatocytes (panels *a* and *b*) were treated with PIA (138 μM) and 14-day-old embryos (panels *c* and *d*) were injected with glutethimide (GT; 1 mmol/kg of embryo wet wt. [7]). Levels of ALA synthase (○) and PB_1 *P*-450 (●) mRNAs (panels *a* and *c*) were expressed as fmol of mRNA/mg of total RNA. ALA synthase enzyme activity (□, panels *b* and *d*) was expressed as either nmol of ALA formed/h per mg of protein (cultures) or nmol of ALA formed/h per g of tissue (embryos). Cytochrome *P*-450 (■, panels *b* and *d*) was expressed as pmol of cytochrome *P*-450/mg of microsomal protein. Values in panels (*a*), (*b*) and (*c*) are the mean ± S.D of three samples and values in panel (*d*) are the mean of several determinations of samples pooled from four embryo livers each for ALA synthase and *P*-450 measurements.

Table 1. Effect of inhibitors of haem biosynthesis on drug induction of ALA synthase and PB_1 *P*-450 in chick embryo hepatocytes *in vivo* and in culture

Chick embryo hepatocyte cultures were treated with combinations of desferrioxamine (Des, 152 μM), PIA (138 μM) and succinylacetone (SA, 50 μM) for 5 h. Chick embryos (14-day-old) were treated with combinations of Des (1.5 μmol/kg embryo wet wt.), DDC (96 μmol/kg), PIA (1 mmol/kg) and glutethimide (GT, 1 mmol/kg) for 7 h.

		ALA synthase mRNA		ALA synthase activity		PB_1*P*-450 mRNA	
Experiment	Treatment	Concentration*	Fold increase†	Specific activity‡	Fold increase†	Concentration*	Fold increase†
Culture A	Control	1.2±0.1	–	0.32±0.01	–	16.7±2.1	–
	Des	1.7±0.4	1.4	0.32±0.01	–	14.9±1.2	–
	PIA	9.9±1.5	8.3	0.62±0.08	1.9	141.5±8.5	8.5
	PIA+Des	10.8±0.8	9.0	1.27±0.18	4.0 (3.2)	134.9±17.4	8.1
Culture B	Control	1.1±0.1	–	0.30±0.05	–	19.0±1.0	–
	SA	1.6±0.3	1.5	0.49±0.07	1.6	17.4±0.9	–
	PIA	14.1±0.9	12.8	0.93±0.04	3.1	88.4±0.6	4.7
	PIA+SA	28.6±1.8	26.0 (2.0)	2.78±0.19	9.3 (3.0)	90.4±8.4	4.8
Embryo	Control	0.9±0.1	–	40	–	1.4±0.2	–
	Des	2.7±0.8	3.1	50	1.2	3.8±0.5	2.7
	DDC	1.8±0.3	2.1	80	1.8	1.0±0.3	–
	PIA	28.2±4.8	32.4	240	5.6	87.5±5.0	63.3
	PIA+Des	81.4±15.1	93.3 (2.8)	480	11.3 (1.7)	87.4±11.6	63.2
	PIA+DDC	60.9±11.9	69.7 (2.1)	680	16.2 (2.2)	82.2±10.7	59.5
	GT	13.4±3.4	15.4	160	3.7	58.9±7.9	42.6
	GT+Des	63.9±18.2	73.2 (4.4)	340	8.0 (1.5)	54.9±6.3	39.7
	GT+DDC	55.4±7.9	63.5 (4.0)	660	15.6 (2.8)	52.7±3.4	38.2

* fmol ALA synthase or PB_1 *P*-450 mRNA/mg of total RNA, mean±S.D., $n = 3$.

† Fold increase over control. Numbers in parentheses represent the fold synergistic increase in induction of the drug combination relative to the additive response of the induction by either drug alone, as calculated by: [(drug A+drug B) −control]÷[(drug A−control)+(drug B−control)].

‡ Cultures: nmol of ALA formed/h per mg of protein, mean±S.D., $n = 3$. Embryos: nmol of ALA formed/h per g of liver (wet wt.), mean of two to three determinations of samples pooled from four embryos.

nuclease-resistant counts (complete reaction conditions but without RNA); precipitable counts (complete reaction conditions, but without RNA or S1 enzyme added); and total counts (probe added directly to scintillation vial). S1-resistant counts (160–200 c.p.m.) were approx. 3-fold above background, and precipitable counts [(5–8) × 10^4 c.p.m.] were approx. 50–80% of total counts. Under the conditions described above, this assay provides a linear, quantitative measure of specific mRNA concentrations in total cellular RNA samples. We performed the assay at a cDNA probe:specific mRNA ratio of 10:1 to 100:1 (determined experimentally); the assay was linear only when the concentration of cDNA probe was present in at least a 5-fold molar excess over the concentration of its specific mRNA. At lower cDNA:mRNA ratios, the probe became limiting and the assay was non-linear [15,17,18].

RESULTS

Time course of ALA synthase and PB_1 *P*-450 induction

The time course of ALA synthase and cytochrome *P*-450 induction was determined in cultured hepatocytes treated with PIA and in embryos treated with glutethimide. Both drugs induced rapid and simultaneous increases in ALA synthase and PB_1 *P*-450 mRNAs, ALA synthase enzyme activity and total cytochrome *P*-450 (Fig. 1). In hepatocyte cultures, ALA synthase and PB_1 *P*-450 mRNA levels were increased significantly within 15 min of drug administration. ALA synthase mRNA was maximal at 1 h, whereas, PB_1 *P*-450 mRNA increased continuously for up to 5 h after drug administration. In chick-embryo liver *in vivo*, both mRNAs were increased within 30 min of drug administration. ALA synthase mRNA was maximally induced by approx. 70-fold over control at 3 h, and PB_1 *P*-450 mRNA was maximally induced by approx. 290-fold over control at 5 h. Induction of ALA synthase enzyme activity and total *P*-450 lagged behind induction of each respective mRNA by 1 to 2 h in both cultured hepatocytes and in chick embryo liver, but followed analogous time courses in each system.

Effect of inhibitors of haem biosynthesis on ALA synthase and cytochrome *P*-450 induction

Many studies have shown that treatment of chick-embryo hepatocytes with a combination of an inducer of ALA synthase plus an inhibitor of haem biosynthesis results in a synergistic induction of ALA synthase enzyme activity above that induced by either compound alone [1,2,19–23]. To determine whether synergistic induction of ALA synthase occurred at the level of mRNA, ALA synthase mRNA was measured in embryo liver or in hepatocyte cultures treated with the inducing drugs, glutethimide or PIA, alone or in combination with inhibitors of haem biosynthesis, DDC, desferrioxamine or succinylacetone [1,2,24,25]. Table 1 summarizes the results of studies on the induction of ALA synthase and PB_1 *P*-450 mRNAs and ALA synthase activity by

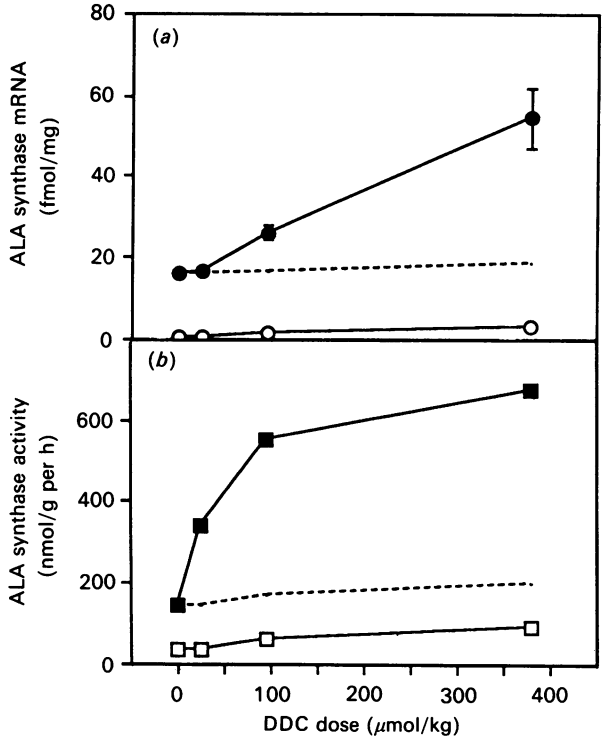

Fig. 2. Dose-dependent effect of DDC on ALA synthase induction in chick embryo liver *in vivo*

Embryos (14-day-old) were treated with DDC with (●,■) or without (○,□) glutethimide (1 mmol/kg) for 7 h. ALA synthase mRNA (panel *a*) was expressed as fmol of mRNA/mg of total RNA. ALA synthase enzyme activity (panel *b*) was expressed as nmol of ALA formed/h per g of tissue. Values in panel *a* are the mean ± S.D of three samples, and values in panel *b* are the mean of several determinations of a pool of four embryo livers. The dotted line represents the theoretical values predicted if glutethimide and DDC administered together were to give a simple additive response based on their individual effects.

combinations of inducing drugs and inhibitors of haem biosynthesis in chick-embryo hepatocytes *in vivo* and in culture. The doses of PIA and glutethimide used were determined to be maximal for induction of ALA synthase and PB_1 *P*-450 mRNAs at these time points (results not shown). Doses of the various inhibitors of haem biosynthesis were chosen for their ability to maximally synergize the induction of ALA synthase activity when administered in combination with the inducing drugs (results not shown).

Inhibitors of haem biosynthesis when given alone caused only small (2- to 3-fold) increases in ALA synthase mRNA and activity (Table 1). All combinations of an inducer plus an inhibitor of haem biosynthesis synergistically increased the induction of ALA synthase enzyme activity above that of the inducer alone. Most combinations also caused a synergistic induction of ALA synthase mRNA induction at the high doses used (Table 1). However, a lower dose of DDC which also synergistically increased drug-inducible ALA synthase activity did not significantly increase ALA synthase mRNA induction (Fig. 2). Similar results were obtained with the other inhibitors of haem biosynthesis (results not shown). Table 1 also shows that inhibitors of haem biosynthesis had no effect on induction of PB_1 *P*-450 mRNA by the inducing drugs, and had little or no effect on basal PB_1 *P*-450 mRNA levels in the absence of an inducing drug. DDC has previously been reported to induce *P*-450 protein [24] and PB_1 *P*-450 mRNA [26] in chick embryo liver; however, these studies used higher (4- to 8-fold) doses of DDC and much longer treatment times.

Effect of haem on ALA synthase and cytochrome *P*-450 induction

The dose-dependent effect of haem on induction of ALA synthase and cytochrome *P*-450 was investigated in cultured hepatocytes which had also been treated with PIA plus desferrioxamine (Fig. 3). Induction of ALA synthase activity by PIA plus desferrioxamine was inhibited 87% by 0.3 μM-haem and completely inhibited by 0.76 μM- and 2.0 μM-haem. Induction of ALA synthase mRNA by PIA plus desferrioxamine was only partially inhibited by haem, but the inhibition was no greater than 50% even at the highest dose. This treatment still resulted in a 4-fold induction of mRNA by PIA plus

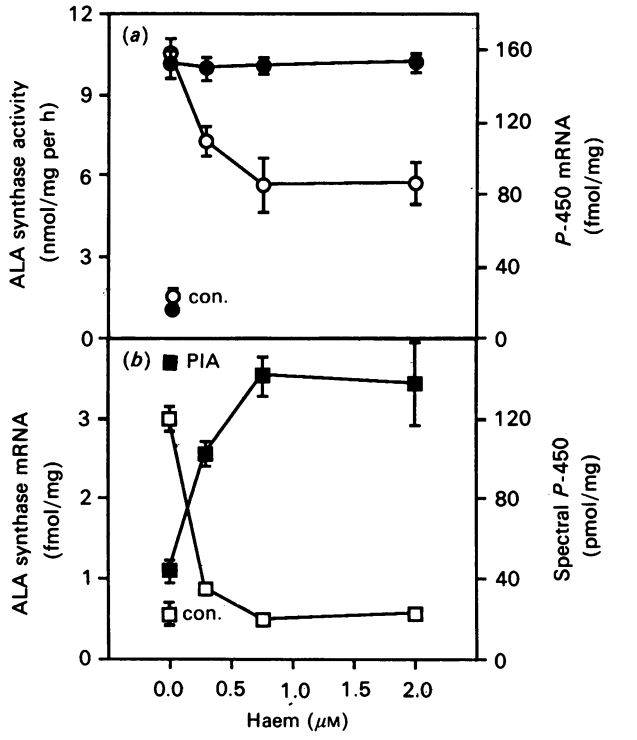

Fig. 3. Effect of haem on ALA synthase and cytochrome P-450 induction in cultured chick-embryo hepatocytes

Cultures were treated with PIA (138 μM) plus desferrioxamine (152 μM) in the absence or presence of haem for 5 h. ALA synthase (○) and PB_1 *P*-450 (●) mRNAs (panel *a*) were expressed as fmol of mRNA/mg of total RNA. ALA synthase enzyme activity (□, panel *b*) was expressed as nmol of ALA formed/h per mg of protein. Cytochrome *P*-450 (■, panel *b*) was expressed as pmol of *P*-450/mg of protein. Values are the mean ± S.D. of three samples. The data points labelled 'con.' represent values from non-induced cultures, and the data point labelled 'PIA' represents values from cultures treated for 5 h with PIA alone.

desferrioxamine, relative to control, versus an 8-fold induction in the absence of haem. Similarly, Ades and co-workers [15,18] previously found that high doses of haem suppressed ALA synthase mRNA by approx. 50% while completely suppressing drug induction of ALA synthase activity. The data in Fig. 3, in conjunction with the data in Fig. 2 and Table 1, indicate that changes in cellular haem levels can modulate both basal and drug-inducible ALA synthase mRNA expression by approx. 2-fold. These results predict the presence of a haem-responsive element within the controlling region of the ALA synthase gene.

The induction of PB_1 *P*-450 mRNA by PIA plus desferrioxamine was unaffected by addition of haem (Fig. 3). There was no apparent increase in holo-cytochrome *P*-450 following PIA plus desferrioxamine induction in the absence of added haem, since the haem biosynthesis inhibitor, desferrioxamine, presumably prevented conversion of cytochrome *P*-450 apo-protein to holo-protein. Addition of 0.3 μM- or 0.76 μM-haem together with PIA plus desferrioxamine, though abolishing ALA synthase activity, only partially restored the spectrally-detected increase in cytochrome *P*-450 to approx. 51% and 89% that induced by PIA alone, indicating that this range of extracellular haem concentration was available for incorporation into cytochrome *P*-450.

Effect of cycloheximide on ALA synthase and cytochrome *P*-450 induction

According to the currently accepted model of ALA synthase regulation, synthesis of cytochrome *P*-450 apoprotein is obligatory in the drug-mediated induction of ALA synthase mRNA [1,2]. To determine whether protein synthesis is required for drug induction of ALA synthase mRNA, the effect of cycloheximide on this induction was investigated in cultured hepatocytes. Maximal suppression of protein synthesis (> 85% as measured by ^{14}C-leucine incorporation) was attained by treatment of cultured hepatocytes for 15 min with 1.8 μM-cycloheximide (results not shown). Therefore, cultures were treated with cycloheximide for 15 min prior to the addition of other chemicals.

Cycloheximide did not block induction of ALA synthase mRNA by PIA (Fig. 4). In fact, cycloheximide caused a 2-fold increase in induction of ALA synthase mRNA by PIA. Cycloheximide alone had little or no effect on basal ALA synthase mRNA levels in this experiment. However, in a replicate experiment, cycloheximide caused a small (2- to 3-fold) increase in basal ALA synthase mRNA level (results not shown). These results suggest the presence of a repressor for the ALA synthase gene, which might also be responsible for controlling a putative haem-responsive element which modulates expression of ALA synthase mRNA. Fig. 4 shows that in the absence of cycloheximide, haem repressed the PIA induction of ALA synthase mRNA by approximately 50% whereas in the presence of cycloheximide, haem had no effect on the induction of ALA synthase mRNA by PIA. Cycloheximide did not prevent the entry of haem into the cells as shown by the lack of any effect of cycloheximide on conversion of exogenously added haem to biliverdin (results not shown). These results suggest that the ALA synthase repressor is responsive to haem.

Treatment of cultures with cycloheximide alone

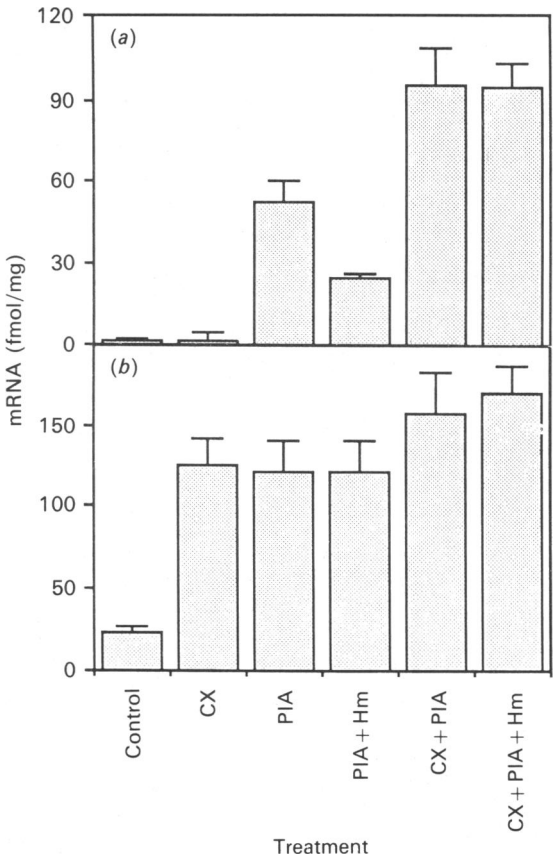

Fig. 4. Effect of cycloheximide on ALA synthase and cytochrome PB_1 *P*-450 mRNA induction in cultured chick-embryo hepatocytes

Cultures were treated with PIA (138 μM) for 60 min in the presence or absence of haem (Hm, 2 μM), and either with or without a cycloheximide treatment (CX, 1.8 μM). Cycloheximide was administered 15 min prior to the addition of PIA or haem. Cells were also treated with cycloheximide alone for 75 min. Control cells received no treatment (control). ALA synthase (panel *a*) and PB_1 *P*-450 (panel *b*) mRNAs were expressed as fmol of mRNA/mg of total RNA. Values are the mean ± S.D. of three samples.

increased PB_1 *P*-450 mRNA levels to a similar extent to treatment with PIA alone, and cycloheximide did not block induction of PB_1 *P*-450 mRNA by PIA (Fig. 4). These results suggest the presence of a labile protein repressor for the PB_1 *P*-450 gene. Haem had no effect on the induction of PB_1 *P*-450 mRNA by PIA either in the presence or absence of cycloheximide.

DISCUSSION

In the work described in this paper, we tested the currently held model for co-ordinate regulation of ALA synthase and cytochrome *P*-450. ALA synthase enzyme activity is inducible in the chick-embryo liver by drugs such as phenobarbital that also cause induction of at least two cytochrome *P*-450 mRNAs and the appearance of an approx. 50000 Da cytochrome *P*-450 isoenzyme [14,16,27,28]. Induction of ALA synthase activity is inhibited by haem, and inhibitors of haem biosynthesis when given in combination with an inducing drug

cause a synergistic induction of ALA synthase activity [1,2,6,19]. The current model proposes that basal and inducible ALA synthase activity is regulated solely by variations in haem concentrations in a 'free haem pool'. Thus, it is proposed that inducers of cytochrome *P*-450 indirectly induce ALA synthase by decreasing this free haem pool [1,2]. Several predictions can be made from this model: (1) following drug treatment, cytochrome *P*-450 mRNA will be induced before ALA synthase mRNA; (2) drug-induced synthesis of cytochrome *P*-450 apoprotein will be obligatory for the induction of ALA synthase mRNA; (3) in the absence of inducing drugs, inhibitors of haem biosynthesis should deplete haem sufficiently to result in induction of ALA synthase mRNA; and (4) addition of haem should prevent drug induction of ALA synthase mRNA.

Our results are incompatible with all the predictions from this model. We employed a sensitive, quantitative solution-hybridization assay to measure changes in steady-state levels of ALA synthase and PB_1 *P*-450 mRNAs. This enabled us to examine very early events in the induction process. We found that both ALA synthase and PB_1 *P*-450 mRNAs increased rapidly and simultaneously following treatment of hepatocyte cultures with PIA or of embryos with glutethimide. Inhibition of protein synthesis by cycloheximide did not prevent induction of ALA synthase mRNA by drugs; in fact, the induction was increased by cycloheximide treatment. These results indicate that synthesis of the apoprotein for cytochrome *P*-450 was not required for the induction of ALA synthase mRNA by drugs. This conclusion is compatible with earlier indirect studies in which ALA synthase mRNA was apparently induced in the presence of cycloheximide [29,30]. Furthermore, we found that inhibitors of haem biosynthesis when administered alone caused only small increases in ALA synthase mRNA levels, relative to the large increases observed following administration of inducing drugs alone. Similarly, many previous investigators have shown that inhibitors alone caused only small increases in ALA synthase enzyme activity [6,21–25]. These results are also incompatible with a model in which haem levels are the principal regulatory component in the induction of ALA synthase mRNA by drugs. In agreement with Ades *et al.* [15,18], we found that doses of haem which completely inhibited induction of ALA synthase enzyme activity only decreased drug induction of ALA synthase mRNA by 50%. Even at the highest dose of haem used, PIA still induced a 4-fold increase in ALA synthase mRNA levels. These results suggest that the decrease in drug-inducible ALA synthase activity following haem treatment is not primarily due to effects on mRNA concentrations.

Early studies with chick embryo hepatocytes showed that haem acted as rapidly as cycloheximide to inhibit induction of ALA synthase activity [29–31]. Haem was subsequently shown in several laboratories to inhibit translocation of newly synthesized enzyme from the cytosol to the mitochondrion (see reviews [1,2]). Our results indicated that this post-translational site is the principal site for the regulation of ALA synthase activity by haem, a point not previously emphasized. Lower doses of inhibitors of haem biosynthesis synergistically increased induction of ALA synthase enzyme activity by drugs, but did not increase ALA synthase mRNA. However, higher doses of inhibitors synergistically increased ALA synthase mRNA induction by drugs, but caused no further increase in ALA synthase enzyme activity. Thus, increases in ALA synthase mRNA may occur under conditions in which there are no parallel increases in enzyme activity, and vice versa. These results suggest that there is no direct correlation between induced levels of ALA synthase mRNA and enzyme activity.

Our results are more compatible with the following scheme for the regulation of hepatic ALA synthase induction by haem. Haem regulates ALA synthase primarily at the site of translocation of newly-synthesized enzyme into the mitochondrion. Inducing drugs act at the transcriptional level to increase ALA synthase mRNA. Thus, the amount of enzyme activity in the mitochondrion is dependent both on the amount of mRNA and on the level of cellular 'free haem', which can be affected by either inhibitors of haem synthesis or exogenously added haem. Treatments which changed haem levels also modulated both basal and drug inducible ALA synthase mRNA levels by approximately two-fold. However, the role of such modulation in the overall regulation of hepatic ALA synthase expression is not clear. Aspects of this model have been proposed previously [6] but could not be tested without direct assays of ALA synthase mRNA levels. It should be noted that the solution hybridization assay we employed measures steady-state mRNA levels rather than transcription rates. We are currently performing run-off transcription assays to confirm that the changes in steady-state mRNA levels we observed with our treatments were principally a result of changes in the transcription rates of the ALA synthase and PB_1 *P*-450 genes. Preliminary experiments indicate that a 1 h-glutethimide treatment of chick embryos results in a substantial increase in the transcription rate of the hepatic ALA synthase gene (J. W. Hamilton, unpublished results). Similarly, Srivastava *et al.* [32] have recently reported that a 4 h-treatment of rats with 2-allyl-2-isopropylacetamide caused a significant increase in the rate of hepatic ALA synthase transcription.

Our experiments with cycloheximide produced two new results concerning regulation of ALA synthase mRNA expression. Cycloheximide increased the drug-mediated induction of ALA synthase mRNA. In addition, cycloheximide abolished the suppressive effect of haem on induction of ALA synthase mRNA by drugs. These results indicate the presence of a labile repressor protein which modulates ALA synthase mRNA expression and which is sensitive to haem. This labile repressor may interact with a putative haem-responsive element which is responsible for modulating expression of the ALA synthase gene.

Our results are also relevant to current models for regulation of PB-inducible cytochrome *P*-450s. Inducing drugs caused a rapid and substantial increase in PB_1 *P*-450 mRNA levels, and the time-course was similar to that of ALA synthase mRNA induction. We found no evidence that PB_1 *P*-450 mRNA expression was modulated by haem, since neither exogenously added haem nor inhibitors of haem biosynthesis affected basal or drug-inducible PB_1 *P*-450 mRNA expression. In contrast, Padmanaban and co-workers [33], on the basis of treatments of rats with high doses of cobalt and aminotriazole, have concluded that the PB-inducible rat liver *P*-450 IIB1+2 genes (previously called *P*-450b+e) require haem for expression. This apparent discrepancy with our results may be due either to species differences

or the high concentrations of inhibitors they used. We found that induction of PB_1 *P*-450 mRNA by drugs also did not require protein synthesis, since cycloheximide treatment did not inhibit this induction. In fact, cycloheximide treatment alone induced PB_1 *P*-450 mRNA to a similar extent as PIA alone, suggesting the presence of a labile protein repressor for the PB_1 *P*-450 gene.

The mechanism of induction of the cytochrome *P*-450 IIB and IIC isoenzymes by PB-like drugs is not known, although these drugs have been shown to increase transcription of *P*-450 IIB mRNAs [3,34,35]. There is presently no evidence for a PB receptor analogous to the cytosolic receptor which mediates induction of isoenzymes in the *P*-450 IA sub-family by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3-methylcholanthrene and other polycyclic aromatic hydrocarbons [5]. Our results suggest that drugs such as PIA and glutethimide may induce PB_1 *P*-450 mRNA by removing a protein repressor of the PB_1 *P*-450 gene. However, the mechanism of induction may be quite different in mammals, since analogous experiments indicate that cycloheximide alone does not induce *P*-450 IIB1 + 2 in rat hepatocytes *in vivo* or in hepatoma cultures and, furthermore, inhibits the induction of *P*-450 IIB1 + 2 mRNAs by PB [36,37]. Similarly, Whitlock and co-workers [5,38] have shown that cycloheximide treatment alone does not induce the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible *P*-450 IA1 gene (previously called P_1-450) in mouse hepatoma cells. It is possible that cycloheximide itself acts as a PB-like inducer of cytochrome *P*-450 mRNA synthesis. However, since low concentrations of cycloheximide were used, relative to glutethimide and PIA, and since cycloheximide alone did not increase ALA synthase mRNA, we conclude that cycloheximide probably does not act as a direct inducer of transcription.

In conclusion, we found that ALA synthase and PB_1 *P*-450 transcription are simultaneously induced by PB-type drugs. We found no evidence to support the current model that ALA synthase is regulated solely by haem. We observed that ALA synthase enzyme activity was very sensitive to changes in haem compared with ALA synthase mRNA expression. Our results are consistent with a model in which the principal site of ALA synthase regulation by haem at physiologically relevant concentrations occurs at a post-transcriptional site, presumably at the post-translational step(s) involving transport across the mitochondrial membrane [1,2]. Our results also indicate the presence of a repressor protein for the PB_1 *P*-450 gene, and a separate repressor protein for the ALA synthase gene that is sensitive to haem. These findings suggest that the ALA synthase and PB_1 *P*-450 genes are regulated by different mechanisms.

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