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ELEVATION OF δ-AMINOLEVULINIC ACID SYNTHASE AND CYTOCHROME PB_1 P450 MESSENGER RNA LEVELS BY DIHYDROPYRIDINES, DIHYDROQUINOLINES, SYDNONES, AND *N*-ETHYLPROTOPORPHYRIN IX

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Abstract—A series of compounds that increase the activity of δ-aminolevulinic acid synthase (ALAS) in chick embryo hepatocyte cultures were studied for their effects on steady-state levels of mRNA for ALAS and phenobarbital-inducible cytochrome PB_1 P450. *N*-Ethylprotoporphyrin IX (N-EtPP), which is believed to lower heme levels by inhibition of ferrochelatase (FC), had little effect on steady-state ALAS mRNA levels. 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-isobutylpyridine (4-isobutyl DDC), which is believed to lower heme levels by repetitive destruction of the heme moiety of cytochrome P450, increased steady-state levels of ALAS mRNA by approximately 2-fold. 3,5-Diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethyl DDC) which inhibits FC activity and destroys the heme moiety of cytochrome P450, increased ALAS mRNA levels approximately 4-fold. A combination of N-EtPP and 4-isobutyl DDC produced a synergistic increase in ALAS mRNA levels to approximately 6-fold over control levels. The synergistic increase in ALAS activity observed previously with this combination can be explained, at least in part, by a synergistic increase in ALAS mRNA levels. Other porphyrinogenic agents, which function as mechanism-based inactivators of cytochrome P450 and elevate ALAS activity, were found to elevate ALAS mRNA. These compounds included 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS), 2,4-diethyl-2-methyl-1,2-dihydroquinoline (DMDQ), and 2,2,4-trimethyl-1,2,dihydroquinoline (TMDQ). The elevation of ALAS mRNA by these porphyrinogenic agents is probably due to their lowering of cellular heme levels by a combination of ferrochelatase inhibition and repetitive destruction of the heme moiety of cytochrome P450. The lowering of heme levels should result in an enhancement of ALAS mRNA half-life as it has been demonstrated by others that heme shortens the half-life of ALAS mRNA. It was of interest that some of these drug treatments also caused an elevation in steady-state levels of cytochrome PB_1 P450 mRNA; the exception was TTMS, which along with its analogue 3-(2-phenylethyl)-4-methylsydnone (PEMS), did not alter cytochrome PB_1 P450 mRNA levels. Increases in steady-state levels of cytochrome PB_1 P450 mRNA subsequent to increases in steady-state levels of ALAS mRNA were observed with 4-ethyl DDC, 4-isobutyl DDC, DMDQ, and TMDQ. The data obtained with N-EtPP and a combination of N-EtPP and 4-isobutyl DDC on cytochrome PB_1 P450 mRNA levels do not support the contention that heme functions as a positive regulator of cytochrome P450 gene expression.

Hepatic heme synthesis is regulated by the first enzyme in the heme biosynthetic pathway, δ-aminolevulinic acid synthase (ALAS)¶ (EC 2.3.1.37), via feedback repression by the end-product heme [1]. It has been postulated that heme regulates ALAS activity through a free heme pool in cells; free heme is heme which is not bound by cellular components [1]. As the level of free heme decreases, the activity of ALAS is increased and vice versa. Heme has been reported to manifest this control in four ways: (a) by inhibiting transcription of mRNA for ALAS [2, 3]; (b) by decreasing the half-life of ALAS mRNA [4,**]; (c) by inhibiting translation of ALAS [5, 6]; and (d) by slowing the transfer and conversion of pre-ALAS from the cytosol to mature ALAS in the mitochondria [7, 8], where it participates in heme synthesis. However, there is conflicting evidence for the role of heme in each of these processes with the exception of the translocation step and the shortening of the half-life of ALAS mRNA.

A compound which has proved useful in the study of heme synthesis is 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethyl DDC), which lowers the activity of ferrochelatase (FC) (EC 4.99.1.1) [9]. 4-Ethyl DDC

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¶ Abbreviations: ALAS, δ-aminolevulinic acid synthase; FC, ferrochelatase; 4-ethyl DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine; 4-isobutyl DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-isobutyl-pyridine; N-EtPP, *N*-ethylprotoporphyrin IX; TTMS, 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone; PEMS, 3-(2-phenylethyl)-4-methylsydnone; DMDQ, 2,4-diethyl-2-methyl-1,2-dihydroquinoline; and TMDQ, 2,2,4-trimethyl-1,2-dihydroquinoline.

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causes mechanism-based inactivation of cytochrome P450; during its biotransformation by cytochrome P450, an ethyl radical is released from 4-ethyl DDC which binds to a nitrogen of the heme moiety of cytochrome P450, producing *N*-ethylprotoporphyrin IX (N-EtPP) [10], a potent inhibitor of FC activity [11–13]. 4-Ethyl DDC also produces an induction of ALAS activity [12]; the induction of ALAS activity has been postulated to be due primarily to the inhibition of FC by the *N*-alkylprotoporphyrin [14]. This theory was tested in a previous study by manipulating cellular heme levels in a chick embryo hepatocyte system and observing the effects on the activity of ALAS [15]. Cellular heme levels were lowered in three ways: (1) by inhibiting FC activity with N-EtPP; (2) by alkylating the heme moiety of cytochrome P450 with 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-isobutylpyridine (4-isobutyl DDC), the alkylated heme is released and the free-apocytochrome P450 combines with a new heme moiety [16], thereby removing heme from the regulatory free heme pool; (3) by inhibiting FC activity and producing mechanism-based inactivation of cytochrome P450 with 4-ethyl DDC. It is possible that the induction of cytochrome P450 also contributed to the lowering of heme levels in our studies. We showed that the induction of ALAS by 4-ethyl DDC could not be explained solely by its inhibition of FC activity by N-EtPP [15]. An interesting finding of the previous study was a synergistic effect on ALAS induction by a combination of N-EtPP and 4-isobutyl DDC [15].

The first objective of the present study was to determine whether the synergistic induction of ALAS activity could be explained, at least in part, by changes in steady-state ALAS mRNA levels. To accomplish this, the effects of 4-ethyl DDC, N-EtPP, 4-isobutyl DDC, and N-EtPP + 4-isobutyl DDC on ALAS mRNA levels were assessed over time in a chick embryo hepatocyte culture system.

Sydnones and dihydroquinolines are two groups of compounds which share the mechanism of action of dihydropyridines. 3-[2-(2,4,6-trimethylphenyl)-thioethyl]-4-methylsydnone (TTMS) produces mechanism-based inactivation of chick embryo hepatic cytochrome P450 [17], formation of *N*-vinylprotoporphyrin IX [18] and inhibition of FC activity and induction of ALAS activity [17] in cultured chick embryo hepatocytes; 3-(2-phenylethyl)-4-methylsydnone (PEMS) produces mechanism-based inactivation of cytochrome P450 [17] and formation of N-substituted protoporphyrins [19] but does not alter FC or ALAS activity [17]. Both 2,4-diethyl-2-methyl-1,2-dihydroquinoline (DMDQ) and 2,2,4-trimethyl-1,2-dihydroquinoline (TMDQ) produce mechanism-based inactivation of hepatic cytochrome P450 and accumulation of *N*-alkylprotoporphyrins in the rat; in addition, these compounds cause inhibition of FC activity and induction of ALAS activity in chick embryo hepatocytes [20]. The second objective of this study was to determine if compounds which possess different structures but are believed to act in a similar manner to 4-ethyl DDC to lower heme levels and induce ALAS activity would also produce changes in ALAS mRNA levels similar to those observed with 4-ethyl DDC. To accomplish this, the effects of TTMS, PEMS, DMDQ, and TMDQ on steady-state levels of ALAS mRNA were measured over time in chick embryo hepatocytes.

Most of the xenobiotics which induce ALAS in the liver also induce one or more isozymes of cytochrome P450 [21]. May and coworkers [22] have proposed that porphyrinogenic agents induce the activity of ALAS indirectly by inducing the synthesis of cytochrome P450, thereby producing a drain on the free heme pool. However, Hamilton and coworkers [23] demonstrated that cytochrome P450 induction is not a prerequisite for ALAS induction by the porphyrinogenic agents, 2-propyl-2-isopropylacetamide and glutethimide. The final objectives of this study were 2-fold. (1) To determine if N-EtPP, TTMS, PEMS, DMDQ, or TMDQ induces cytochrome P450, as measured by an increase in steady-state mRNA levels; to accomplish this, the effects of N-EtPP and the DDC analogues, the sydnones, and the dihydroquinolines on steady-state levels of mRNA for cytochrome PB_1 P450 were determined over time in chick embryo hepatocyte culture; and (2) to determine if the dihydropyridines, sydnones, or dihydroquinolines act to increase ALAS activity subsequent to the induction of cytochrome P450; to achieve this objective, the time course of cytochrome PB_1 P450 was compared to the time course of induction of ALAS mRNA. Cytochrome PB_1 P450 is one of two major phenobarbital-inducible isozymes of cytochrome P450 present in chick embryo liver* [25]. While studies have been carried out with non-specific inhibitors of heme biosynthesis such as cobalt chloride and inducers of cytochrome P450 such as propylisopropylacetamide (PIA) and glutethimide, the novel feature of our study was the employment of a highly specific inhibitor of heme biosynthesis, namely N-EtPP, and compounds which are mechanism-based inactivators of cytochrome P450.

MATERIALS AND METHODS

Materials. Guanidine isothiocyanate and guanidine hydrochloride were obtained from ICN Biomedicals (Montreal, PQ). [γ-^{32}P]Adenosine triphosphate (3000 Ci/mmol) was obtained from New England Nuclear (Dupont, Toronto, Ontario). T_4-Polynucleotide kinase and S1-nuclease were obtained from Bethesda Research Laboratories (Burlington, Ontario). All other reagents were molecular biology grade or highly purified (Sigma, St. Louis, MO, or Biorad, Mississauga, Ontario). The DDC analogues were synthesized as described previously [26, 27]. The sydnones and dihydroquinolines were gifts of Dr. P. R. Ortiz de Montellano (Department of Pharmaceutical Chemistry, School of Pharmacy, Liver Center, University of California, San Francisco, CA). *N*-Ethylprotoporphyrin IX was synthesized as described by De Matteis *et al.* [28] and purified by thin-layer chromatography according to the method of McCluskey *et al.* [27]. White Leghorn chick embryos, obtained from Archer's Poultry Farm, Brighton, Ontario, were used at 18 days after fertilization.

* This cytochrome P450 has been designated P450IIH1 according to the nomenclature of Nebert *et al.* [24].

Preparation of RNA. Hepatocytes of 18-day-old chick embryos were obtained as described previously [29] and maintained in 10-cm dishes containing 15 mL of Waymouth 705/1 medium. The medium was changed 24 hr after plating, drugs were added in 95% ethanol (maximum volume, 10 μL/dish), along with an ethanol control, and the cells were incubated for the indicated time points. RNA was isolated by the guanidine isothiocyanate/guanidine hydrochloride precipitation method outlined by Chirgwin *et al.* [30]. The RNA was stored in water at −70° until it was used.

Measurement of specific mRNA levels. Specific mRNAs for either ALAS or cytochrome PB_1 P450 were measured using the solution-hybridization assay outlined by Omiecinski *et al.* [31] as modified by Hamilton *et al.* [23], with the only change being a 9-hr instead of an 18-hr incubation time. Briefly, 10 μg of RNA was incubated for 9 hr with a 100-fold excess of ^{32}P-labelled oligonucleotide probe at 55° as previously described [23]. The sequence for the ALAS probe is $^{5'}$GTAGATCTCACAACCTGGCAGCAT$^{3'}$ [32] and for the cytochrome PB_1 P450 probe is $^{5'}$GTTATTTTCTTCCAGCATCTCAAT$^{3'}$ [25]. These probes have been used previously and have been shown to be specific for their respective mRNAs [23]. The probes were checked and found to be specific for one mRNA species by Northern blotting [33]. Single-strand nucleotides were degraded with S1-nuclease, and the [^{32}P]DNA–RNA hybrid was isolated by trichloroacetic acid (TCA) precipitation and quantitated by liquid scintillation counting as previously described [23]. The controls used were the following: (1) a background of S1-nuclease resistant counts was determined in the absence of RNA and this value was subtracted from all counts; (2) total precipitable counts were determined in the absence of both RNA and S1-nuclease; and (3) the activity of the probe was determined with each experiment [23]. Results are expressed as femtomoles of specific mRNA per milligram of total RNA.

Statistical analysis. A repeated measures design one-way analysis of variance was used to determine whether two means differed significantly from each other ($P \leq 0.05$). If a significant F ratio at the 0.05 level was obtained, a Newman–Keuls test was used to indicate the means which differed significantly from each other.

RESULTS AND DISCUSSION

In a previous study, it was inferred that while the inhibition of FC by 4-ethyl DDC is a more important heme-depleting mechanism than the mechanism-based inactivation of cytochrome P450, the inhibition of FC alone does not explain the induction of ALAS activity observed with 4-ethyl DDC [15]. It was also found that N-EtPP and 4-isobutyl DDC, when administered together in an attempt to mimic the induction of ALAS activity found with 4-ethyl DDC, produce a synergistic increase in ALAS activity [15]. To explain this synergism, it was suggested that (1) the levels of free heme required to exert optimal regulation of ALAS expression could vary, for example as a result of separate nuclear, cytosolic, and mitochondrial divisions of the regulatory free heme pool [15]; (2) inhibition of FC may reduce levels of free heme to the point where only one regulatory mechanism is derepressed; and (3) the addition of the destruction of the heme moiety of cytochrome P450 may lower remaining heme levels to the point where other mechanisms are derepressed, and ALAS activity is able to reach maximal levels.

The present study was designed, in part, to determine if the changes seen in ALAS activity are reflected in changes in ALAS mRNA levels over time. The effect of 4-ethyl DDC on steady-state mRNA levels in cultured chick embryo hepatocytes was determined over a 24-hr period; determinations were made every hour during the first 6 hr to ensure that any early changes were detected. 4-Ethyl DDC significantly increased the steady-state levels of mRNA for ALAS approximately 4-fold; mRNA levels returned to control by 18 hr after treatment (Fig. 1b). This increase in ALAS mRNA was expected, as 4-ethyl DDC both inhibits FC activity and causes mechanism-based inactivation of cytochrome P450, which should lower heme levels, increase the half-life of ALAS mRNA, and thereby increase ALAS mRNA expression [4,*]. Although a statistically significant increase in ALAS mRNA levels was detected at 1, 3, 5, and 12 hr after the administration of N-EtPP, the biological significance of such small changes is questionable (Fig. 1d). 4-Isobutyl DDC, which is believed to lower heme levels by the mechanism-based inactivation of cytochrome P450, significantly increased steady-state ALAS mRNA levels in chick embryo hepatocytes approximately 2-fold; mRNA levels returned to control by 12 hr after treatment (Fig. 1c).

In a previous study it was reported that the combination of N-EtPP and 4-isobutyl DDC produces a synergistic increase in ALAS activity at 12 hr [15]. In the present study this treatment induced a synergistic increase in ALAS mRNA levels to approximately 6-fold over control levels; levels of ALAS mRNA returned to control by 24 hr (Fig. 1e). Therefore it appears that the synergistic increase in ALAS activity observed previously can be explained, at least in part, by a synergistic increase in ALAS mRNA expression. This is probably explained by the lowering of heme levels with both these compounds to an extent considerably greater than that observed with either N-EtPP or 4-isobutyl DDC alone. It is of interest that 4-ethyl DDC, which is believed to lower heme levels by FC inhibition and mechanism-based inactivation of cytochrome P450, had effects on ALAS mRNA levels similar to those produced by the combination of N-EtPP and 4-isobutyl DDC at 9 and 12 hr (compare panels b and e of Fig. 1). The enhanced effect of the combination observed at 18 hr (Fig. 1e) relative to that seen with 4-ethyl DDC (Fig. 1b) could possibly be explained by the maintenance of higher levels of N-EtPP when the combination was used.

Thus it appears that the lowering of cellular heme

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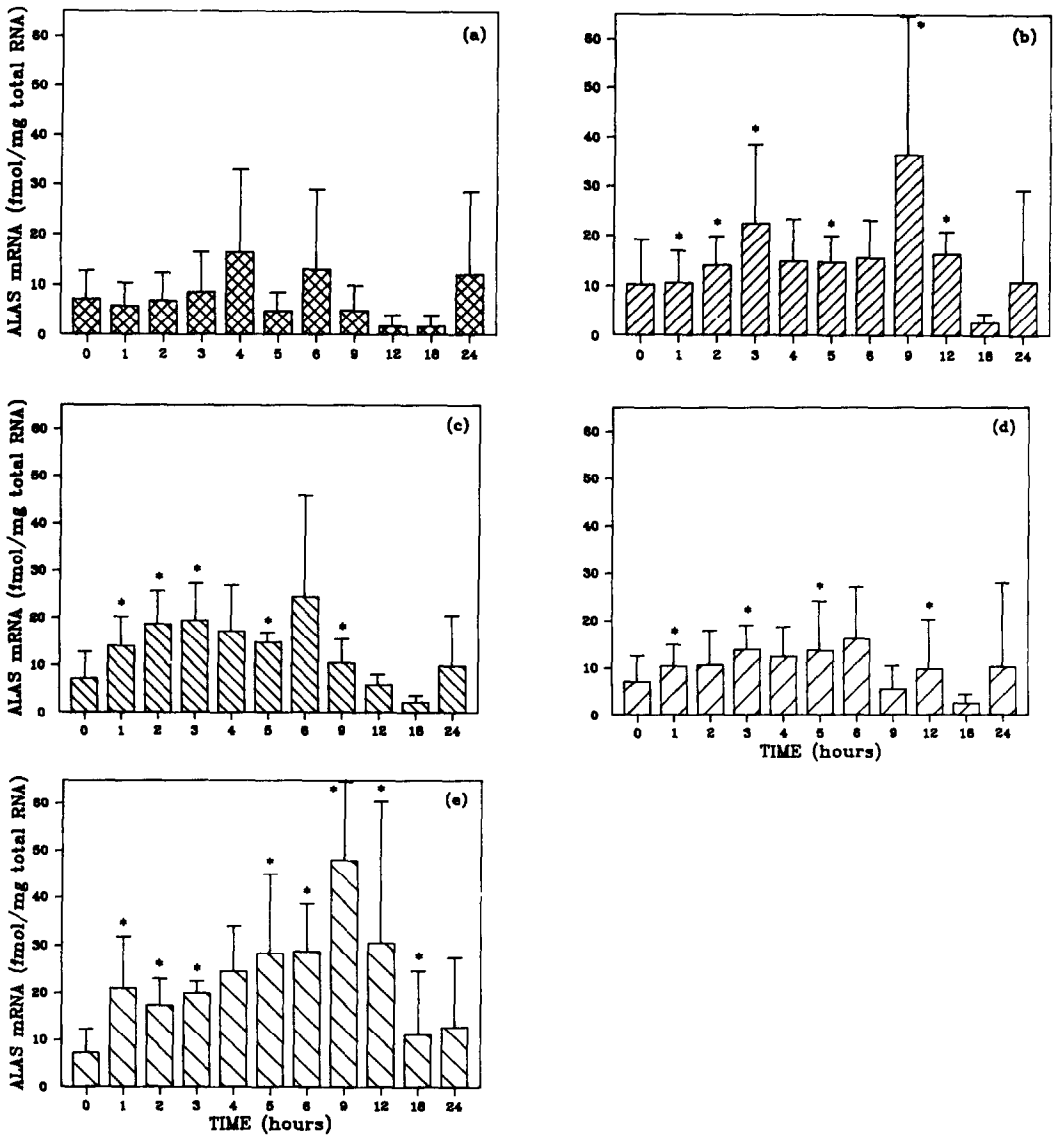

Fig. 1 δ-Aminolevulinic acid synthase mRNA levels in chick embryo hepatocyte culture at various times after the administration of: (a) 95% EtOH; (b) 4 μM 4-ethyl DDC; (c) 4 μM 4-isobutyl DDC; (d) 2 μM N-EtPP; and (e) 2 μM N-EtPP + 4 μM 4-isobutyl DDC. Each point is the mean ± SD of three to four determinations. An asterisk (*) indicates a significant difference from the EtOH control at $P \leq 0.05$.

levels by two mechanisms produces a greater increase in steady-state levels of mRNA for ALAS than does the lowering of heme levels by only one mechanism. This increase in mRNA levels could reflect either an increase in the synthesis of mRNA or a decrease in the degradation of mRNA. Drew and Ades [4] have demonstrated recently that the half-life of ALAS mRNA is decreased by heme in cultured chick embryo hepatocytes. This suggests that the increase in steady-state mRNA levels apparent in this study represents an increase in the stability of ALAS mRNA due to a depletion of cellular heme levels.

In the second part of this study, we were interested in determining the effect on ALAS mRNA of compounds which differ in structure from 4-ethyl DDC but which share a similar mechanism of action. TTMS produces mechanism-based inactivation of cytochrome P450 [17], production of *N*-vinyl-protoporphyrin IX [18], inhibition of FC activity, and induction of ALAS [17], as does 4-ethyl DDC. It was anticipated that, in a manner similar to 4-ethyl DDC, TTMS would increase ALAS mRNA levels; this was found to be the case (Fig. 2b). PEMS, an analogue of TTMS, produces mechanism-based inactivation of cytochrome P450 [17] and formation of N-substituted protoporphyrins [19] but does not alter FC or ALAS activity [17]. The lack of effect of PEMS on ALAS activity, despite the interaction with cytochrome P450, might be explained by the alteration of the heme moiety of cytochrome P450 during the metabolism of PEMS so that the apo-cytochrome P450 cannot be reconstituted with a new heme moiety. Precedent for this exists with the compound 1-aminobenzotriazole, which destroys

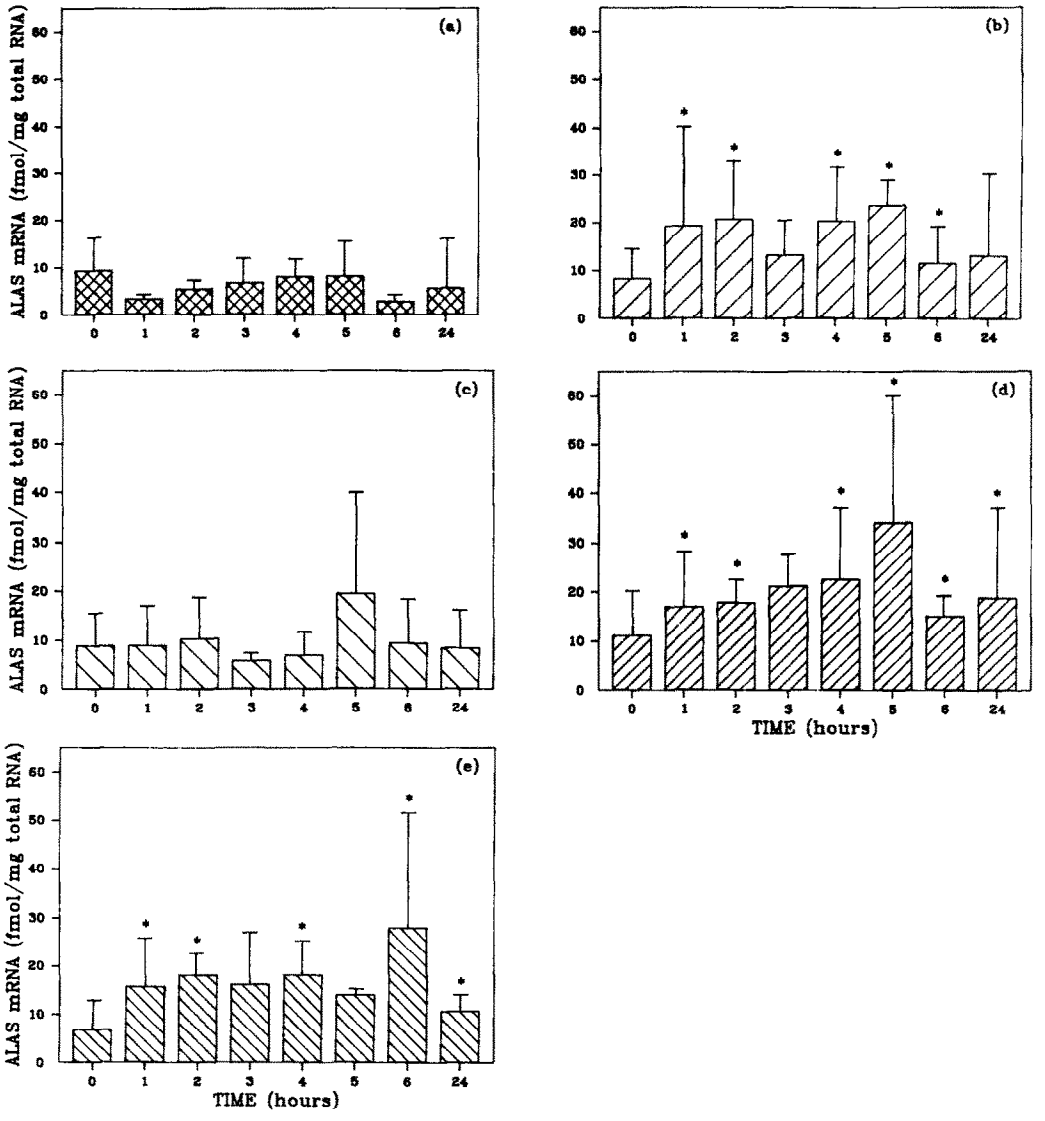

Fig. 2. δ-Aminolevulinic acid synthase mRNA levels in chick embryo hepatocyte culture at various times after the administration of: (a) 95% EtOH; (b) 4 μM TTMS; (c) 4 μM PEMS; (d) 100 μM DMDQ; and (e) 100 μM TMDQ. Each point is the mean ±SD of three determinations. An asterisk (*) indicates a significant difference from the EtOH control at $P \leq 0.05$.

up to 80% of cytochrome P450 in rats, but produces only a slight increase in ALAS activity [34]. It was anticipated that PEMS would not increase the steady-state levels of ALAS mRNA in chick embryo hepatocytes; this was found to be the case (Fig. 2c). It has been suggested that one component of the activation of transcription of the ALAS gene by porphyrinogenic compounds is through a direct effect of drugs on the nucleus. The similarity of the structures of TTMS and PEMS, coupled with the inactivity of PEMS, indicates that, at least with sydnones, this mechanism is inoperative. A second pair of compounds, DMDQ and TMDQ, both produce inactivation of rat cytochrome P450, formation of *N*-alkylprotoporphyrins, lowering of FC activity, and induction of ALAS activity [20], and it was therefore anticipated that, in a manner similar to 4-ethyl DDC and TTMS, they would increase ALAS mRNA levels; this was found to be the case (Fig. 2, panels d and e). An interesting aspect of this increase is that ALAS mRNA levels were still raised significantly at 24 hr after treatment. This suggests a slower rate of biotransformation of the dihydroquinolines.

We were next interested in determining the effects of N-EtPP, TTMS, PEMS, DMDQ, and TMDQ on the levels of mRNA for cytochrome PB_1 P450, as it is not known whether or not these compounds induce cytochrome P450. N-EtPP was found to increase the steady-state levels of cytochrome PB_1 P450 from 2 to 6 hr after treatment; levels returned to control by 24 hr (Fig. 3d). 4-Ethyl DDC, 4-isobutyl DDC, and the combination of 4-isobutyl DDC and N-EtPP produced similar increases in cytochrome PB_1 P450 mRNA (Fig. 3, panels b, c and e), suggesting a similar mechanism of induction of cytochrome P450.

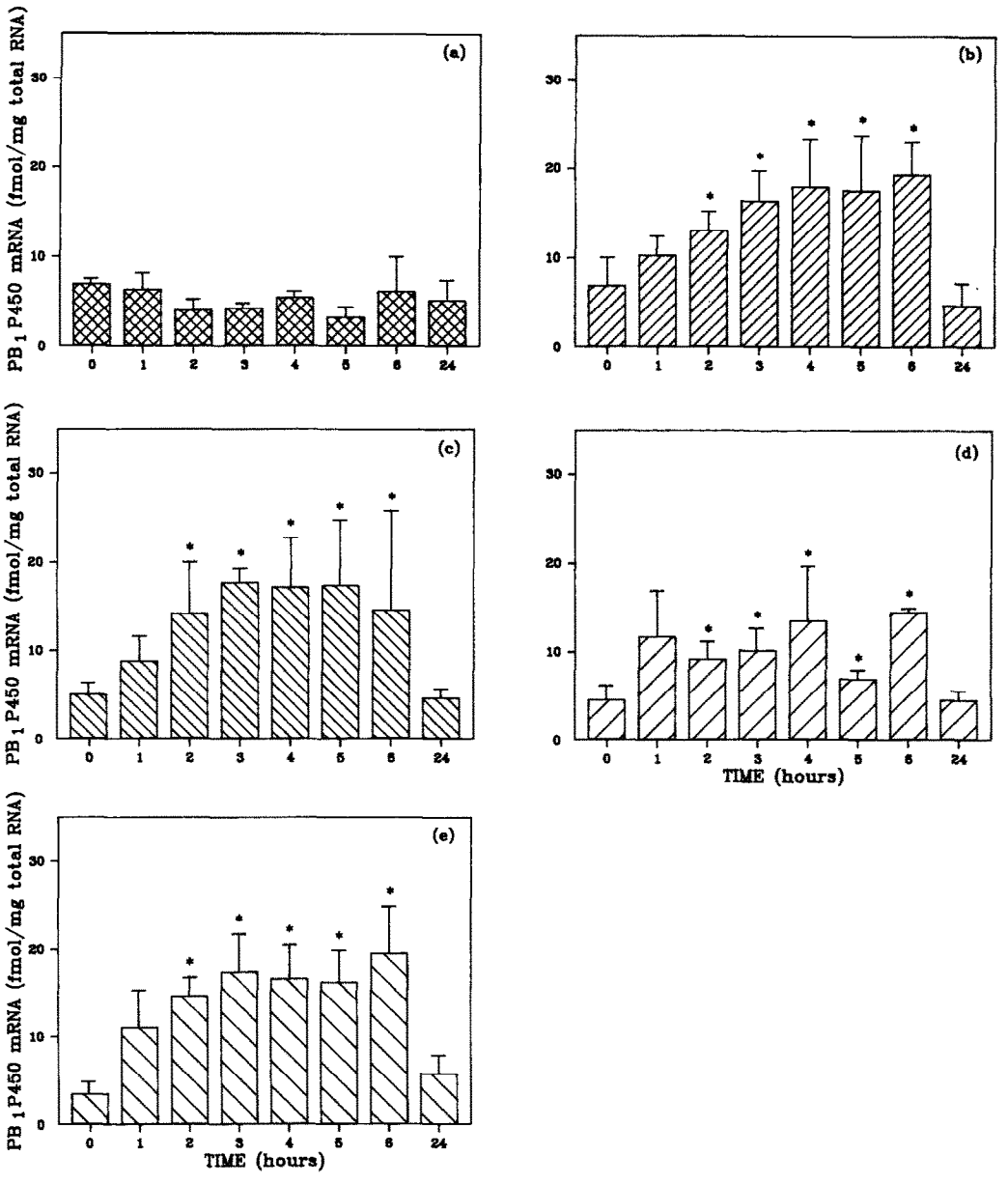

Fig. 3. Cytochrome PB_1 P450 mRNA levels in chick embryo hepatocyte culture at various times after the administration of: (a) 95% EtOH; (b) 4 μM 4-ethyl DDC; (c) 4 μM 4-isobutyl DDC; (d) 2 μM N-EtPP; and (e) 2 μM N-EtPP + 4 μM 4-isobutyl DDC. Each point is the mean ± SD of three to four determinations. An asterisk (*) indicates a significant difference from the EtOH control at $P \leq 0.05$.

At 3 and 5 hr after treatment, the effects of either 4-ethyl DDC, 4-isobutyl DDC, or 4-isobutyl DDC and N-EtPP were greater than that of N-EtPP alone. There was no evidence for any synergistic increase in cytochrome PB_1 P450 mRNA with the combination of N-EtPP and 4-isobutyl DDC. It is of considerable interest that N-EtPP, an inhibitor of heme biosynthesis, did induce the steady-state levels of cytochrome PB_1 P450 mRNA and did not prevent the increase in cytochrome PB_1 P450 mRNA levels observed with 4-isobutyl DDC when the two were administered concurrently in view of the following controversy. Padmanaban and coworkers [35–37] have provided evidence that heme is a positive regulator of cytochrome P450 gene expression using cobalt chloride, 3-amino-1,2,4-triazole and thioacetamide as inhibitors of heme biosynthesis. Srivastava *et al.* [38], using succinyl acetone, a more specific inhibitor of heme biosynthesis, could not confirm that heme functions as a positive regulator of cytochrome P450 gene expression. Our present studies with N-EtPP, a highly specific inhibitor of heme biosynthesis, also do not support the contention that heme functions as a positive regulator of cytochrome P450 gene expression.

It was anticipated that TTMS, which like 4-ethyl DDC produces mechanism-based inactivation of cytochrome P450 [17], would increase the levels of mRNA for cytochrome PB_1 P450; this was not found to be the case. Cytochrome PB_1 P450 levels were

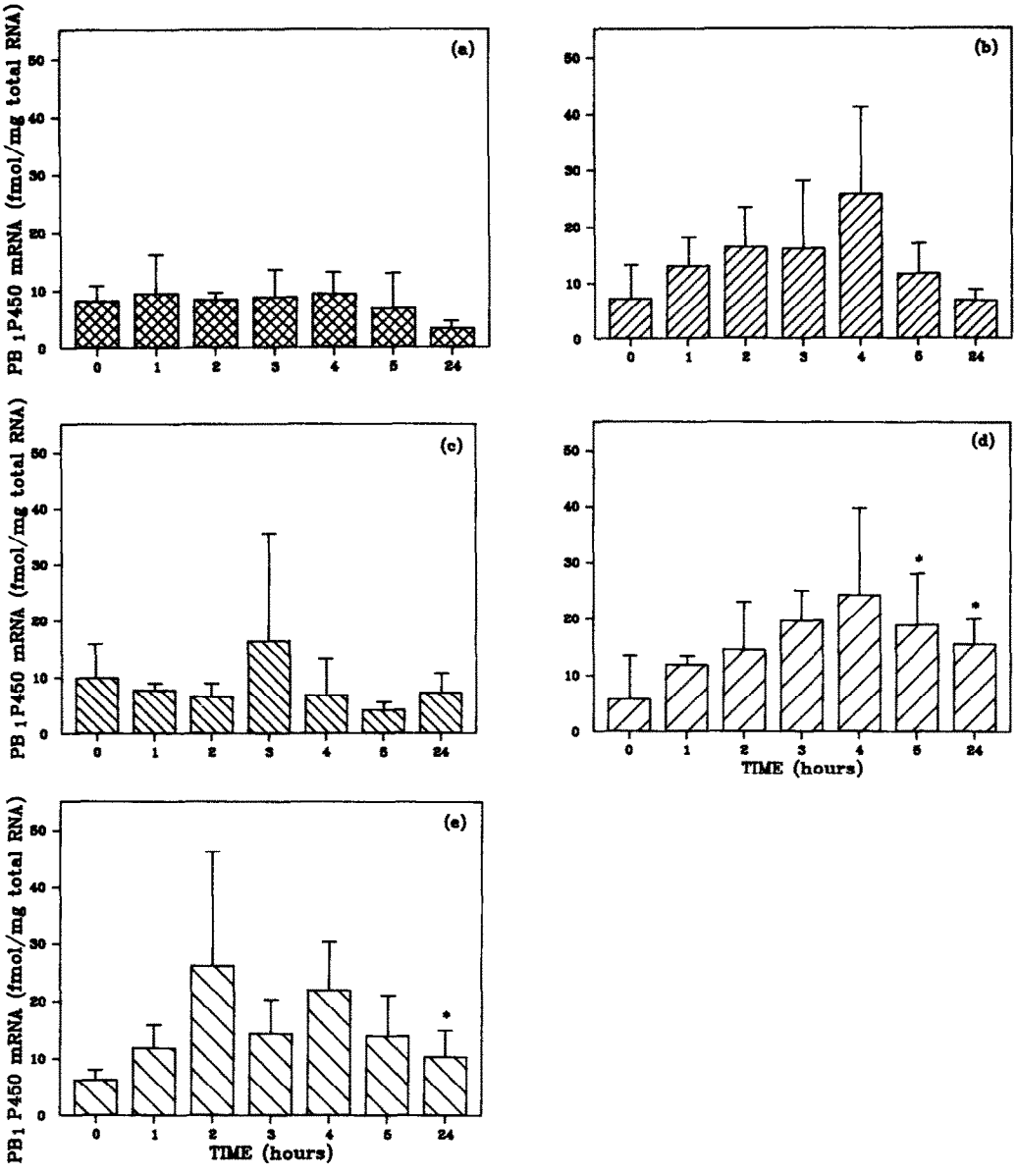

Fig. 4. Cytochrome PB_1 P450 mRNA levels in chick embryo hepatocyte culture at various times after the administration of: (a) 95% EtOH; (b) 4 μM TTMS; (c) 4 μM PEMS; (d) 100 μM DMDQ; and (e) 100 μM TMDQ. Each point is the mean ± SD of three determinations. An asterisk (*) indicates a significant difference from the EtOH control at $P \leq 0.05$.

not increased significantly at any time after treatment with TTMS (Fig. 4b). PEMS, which also causes mechanism-based inactivation of cytochrome P450 [17], did not alter mRNA levels for cytochrome PB_1 P450 at any point after treatment (Fig. 4c). This lack of effect of the sydnones may be explained by the fact that (a) the sydnones do not interact with and induce cytochrome PB_1 P450, the only isozyme of cytochrome P450 measured in this study, or (b) the increase occurred at a time between 5 and 24 hr; these times were not measured in this study. DMDQ and TMDQ, both of which inactivate cytochrome P450 [20], were expected to increase the levels of mRNA for cytochrome PB_1 P450. DMDQ increased cytochrome PB_1 P450 mRNA levels at 5 and 24 hr after treatment (Fig. 4d) and TMDQ increased cytochrome PB_1 P450 mRNA levels at 24 hr after treatment (Fig. 4e).

The final objective of this study was to determine if the induction of cytochrome P450 precedes the induction of ALAS. From Figs. 3 and 4, it is apparent that most compounds which induce the synthesis of mRNA for ALAS also induce the synthesis of cytochrome PB_1 P450 mRNA; the exception is TTMS. However, we found, as did Hamilton *et al.* [23] with 2-propyl-2-isopropylacetamide and glutethimide, that the induction of cytochrome PB_1 P450 mRNA did not precede the induction of ALAS mRNA. With the dihydropyridines and N-EtPP, ALAS mRNA levels were increased by 1 hr after

treatment (Fig. 1, panels b–e), whereas the elevation of cytochrome PB_1 P450 mRNA levels did not occur until 2 hr after treatment (Fig. 3, panels b–e). With the dihydroquinolines, elevation of ALAS mRNA also occurred at 1 hr after treatment but cytochrome PB_1 P450 levels did not increase until considerably later (Figs. 2d, 2e, 4d, and 4e).

In summary, we have shown that 4-ethyl DDC, N-EtPP, 4-isobutyl DDC, and the combination of N-EtPP and 4-isobutyl DDC, which produce enhanced ALAS activity, also cause an increase in the steady-state levels of ALAS mRNA. The synergistic increase in ALAS activity previously observed with the combination of N-EtPP + 4-isobutyl DDC is explained, at least in part, by a synergistic increase in steady-state levels of ALAS mRNA. This is probably due to the fact that heme levels are lowered to a greater extent because two mechanisms for lowering heme levels are operative when the combination of N-EtPP and 4-isobutyl DDC is employed. An additional reason for the synergism in ALAS activity observed with the combination of N-EtPP and 4-isobutyl DDC might be the following: N-EtPP would act at the mitochondrial level to lower heme levels and to facilitate transfer and processing of inactive ALAS from the cytosol into active ALAS in the mitochondria. This factor, coupled with the increased synthesis of ALAS due to the increased stability of the mRNA for ALAS, might explain, at least in part, the synergistic increase in ALAS activity observed previously [15].

We have shown that TTMS, DMDQ, and TMDQ, compounds which possess different structures but act in a similar manner to 4-ethyl DDC in inducing ALAS activity, also produce an increase in the steady-state levels of ALAS mRNA. PEMS, a compound close in structure to TTMS, which does not induce the activity of ALAS, did not increase the steady-state levels of ALAS mRNA. These data suggest that, at least with the sydnones, these compounds induce ALAS activity primarily through an indirect effect on cellular heme levels, as opposed to a direct stimulation of ALAS transcription.

Our data do not support the idea that porphyrinogenic drugs act by first inducing the activity of cytochrome P450, which leads to lowering of the heme pool followed by the induction of ALAS activity. It is possible, however, that the induction of cytochrome P450 leads to a lowering of free heme after the primary induction process and contributes to prolonging the elevated levels of ALAS mRNA and increased ALAS activity.

Finally, our data do not support the idea that heme functions as a positive regulator of cytochrome P450 gene expression, as has been suggested in the literature [35–37].

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