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REGULATION OF PRODUCTION OF EMBRYONIC CHICK LIVER DELTA-AMINOLEVULINATE SYNTHASE: EFFECTS OF TESTOSTERONE AND OF HEMIN ON THE mRNA OF THE ENZYME

Paul D. Drew and Ibrahim Z. Ades

Department of Zoology, University of Maryland, College Park, MD 20742

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The effects of testosterone and of hemin on the concentration of the mRNA of embryonic chick liver ALA synthase were investigated. Using cDNA-RNA liquid hybridization analyses, we determined that testosterone, when injected into the fluid surrounding chick embryos, caused a dose-dependent increase in the concentration of ALA synthase mRNA in liver. Similarly, addition of testosterone (5μg/ml) or of 75μg/ml of allylisopropylacetamide (AIA) into the medium of chick embryo hepatocytes maintained in culture caused an increase in the concentration of ALA synthase mRNA. Hemin (2 or 5μM), when added to the culture medium, inhibited the elevations of ALA synthase mRNA concentration brought on by testosterone and by AIA.

Embryonic chick liver ALA synthase catalyzes the rate limiting step of heme biosynthesis (1). The enzyme is located in the mitochondrial matrix (1) but is synthesized on free cytoplasmic polyribosomes (2) as a larger precursor form (2-4). Liver ALA synthase activity is increased by a variety of xenobiotics (1). Two potent porphyrogenic agents are the barbiturate allylisopropylacetamide (AIA) and the collidine diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridine dicarboxylate (DDC). Numerous steroid hormones and their metabolites can also induce increases in the activity of the enzyme both in embryos (5) and in hepatocyte cultures (6). Among the inducing steroids, 5 βH steroids generally increase the activity of ALA synthase more significantly than their corresponding 5 α H epimers (5, 6). A high 5 βH to 5 αH steroid ratio is associated with acute intermittent porphyria, an inherited liver disorder in humans characterized by elevations in the activity of ALA synthase and overproduction of porphyrins (7). We have been investigating the mechanism of steroid induction of ALA synthase activity in embryonic chick liver. RNA and protein synthesis inhibitors have been known to block steroid induction of the activity of the enzyme (8). This has suggested that steroids might induce de

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novo synthesis of chick liver ALA synthase rather than activate the existing enzyme (8). In this paper, we report that testosterone increased the concentration of ALA synthase mRNA and that hemin inhibited the effect. The measurements on the mRNA were carried out by solution hybridization.

EXPERIMENTAL PROCEDURES

Preparation of hepatocyte cultures: Primary cultures of hepatocytes were prepared from 17-day-old White Leghorn chick embryos as described previously (9). Livers from 40 embryos were used in each preparation. The cells were maintained initially in modified Ham's F-12 medium containing 10% fetal calf serum at 37°C in a humidified incubator (95% air, 5% CO_2). After 16h, the medium was replaced with modified Ham's F-12 medium (9) containing 0.2mg of insulin/liter and 0.25mg of desferoxamine mesylate/ml, and the cells were incubated for 2h at 37°C. The medium was replaced with modified Ham's F-12/insulin medium. Porphyrogenic agents were added to the culture medium at that time, as indicated. AIA and testosterone were added to final concentrations of 75μg/ml and 5μg/ml, respectively, from stock solutions in ethanol. Hemin, when used, was added to the culture medium from a stock solution of 2.5mg of hemin/ml of 15mM KOH in 50% ethanol.

ALA synthase activity determination: The activity of ALA synthase was determined as described by Sassa et al. (6). Protein was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard.

Isolation of RNA: RNA was isolated from chick embryo livers and from hepatocyte cultures as described by Maniatis et al. (11), with modifications. The tissue was homogenized at room temperature in 4M guanidine thiocyanate, 10mM Tris-Cl, pH 7.4, 7% β-mercaptoethanol. Sarcosyl was added to the homogenate to a final concentration of 2%, and the solution was passed twice through an 18-gauge needle. The liver homogenate (8ml) was layered over a 2ml cushion of 5.7M CsCl, 10mM EDTA, pH 7.4, in a centrifuge tube. The gradient was centrifuged at 40,000 rpm in a Beckman SW41 rotor for 22h at 20°C. After centrifugation, the RNA pellet was resuspended in 0.5ml of water and warmed to 65°C for 10min to help dissolve the RNA. The solution was adjusted to 0.2M potassium acetate, and RNA was precipitated with 2 volumes of 95% ethanol at -20°C.

Preparation and radio-labeling of synthetic oligonucleotide: A 24mer oligonucleotide was synthesized using an Applied Biosystem DNA synthesizer (Model 380A). The oligonucleotide was made complementary to bases +795 to +818 of embryonic chick liver ALA synthase mRNA (12). The sequence of the cDNA was 5'-GTAGATCTCACAACCTGGCAGCAT-3'. The oligonucleotide was purified first by electrophoresis through a 20% polyacrylamide preparative gel containing 50% urea, was eluted from a slice of the gel by incubation with 0.5M ammonium acetate, and finally was purified further by passage through a NENSORB^{TM}20 nucleic acid purification cartridge (Dupont). The oligonucleotide was labeled at the 5' end with [^{32}P] as described by Omiecinski et al. (13). The radiolabeled oligonucleotide was then separated from free nucleotides by chromatography on DEAE-Sephacel (14). The resulting cDNA was labeled to a specific activity of 2-4 X 10^6 cpm/pmol.

Solution hybridization analyses: Hybridization assays were performed at 55°C for 18h in 10μl volumes containing 750mM NaCl, 0.2% SDS, 4mM EDTA, 20mM Tris-Cl, pH 7.5, approximately 3000-5000 cpm of[^{32}P]-labeled oligonucleotide, and the indicated amount of liver RNA. (A constant amount of oligonucleotide was used in a given set of analyses). At the end of hybridization, single stranded nucleic acids were digested with 8 units of S_1 nuclease at 37°C for 1h in 1ml of a solution containing 100ug/ml of salmon sperm DNA, 750mM NaCl, 2.8mM

$ZnSO_4$, 70mM sodium acetate, pH 4.5. S_1-resistant material was precipitated for 1h on ice with 200μl of 6M trichloroacetic acid (TCA). Acid-insoluble nucleic acids were collected on 27mm diameter nitrocellulose discs (Schleicher and Schuell). Each disc was washed three times with 10ml of ice-cold 3% TCA in 1% sodium pyrophosphate and then washed three times with ice-cold 95% ethanol. The radioactivity in acid-insoluble nucleic acids was determined using Bray's solution (15). Background cpm of the assay was determined using the same solution hybridization conditions, but no RNA was added to the appropriate blanks prior to hybridization and subsequent digestion with S_1 nuclease. On average, the background was 100 cpm.

RESULTS AND DISCUSSION

The main purpose of this study was to determine the effects of testosterone and of hemin on the concentration of embryonic chick liver ALA synthase mRNA. It had been previously established that testosterone caused an increase in liver ALA synthase activity *in ovo* (16) and in hepatocyte cultures (17). Under our conditions, testosterone caused a significant increase in the activity of the enzyme in embryos (Table 1), but the induction was not as pronounced as that obtained with the more potent porphyrogenic agents AIA and DDC.

The results of cell-free mRNA translations followed by immunoprecipitations of ALA synthase-specific sequences had indicated that the administration of AIA and DDC to chick embryos caused a significant elevation in the concentration of liver ALA synthase mRNA (3). In this study, we measured ALA synthase-specific RNA concentrations more directly using cDNA-RNA solution hybridization analyses. We used in the assays an oligonucleotide (cDNA) synthesized complementary to a

Table I

Effects of testosterone and of AIA plus DDC on ALA synthase activity

Treatment[a]	ALA Synthase Activity (nmoles/h/mg protein)
None	0.79 $\pm$ 0.13
2mg AIA + 4mg DDC	24.3 $\pm$ 4.98[b]
5mg Testosterone	2.18 $\pm$ 0.36[b]

[a] The indicated agent was injected through the air sac membranes of 16-day-old embryos. The embryos were incubated for 18h at 38°C, and then ALA synthase activity was determined in liver homogenates. Three determinations were made for each condition, and livers from 2-6 embryos were pooled for each determination. Values represent means $\pm$ standard errors.

[b] Significantly different from control ($P < 0.05$) as determined by the Student t test.

known sequence of ALA synthase mRNA (12). In preliminary analyses, we tested the solution hybridization by using in the assay RNA from livers of embryos that had been treated with AIA and DDC. In these assays, the radioactivity recovered in material resistant to S_1 nuclease digestion at the end of hybridization varied almost linearly with the amount of RNA added to the reactions, up to 30ug (Fig. 1). Almost no hybrids were detected when RNA from normal, rather than porphyric, embryos was used. Next, we measured the effect of testosterone on the concentration of chick liver ALA synthase-specific RNA levels. Testosterone, when injected into the fluid surrounding the embryos, caused an increase in the concentration of ALA synthase-specific RNA (Fig. 2); this

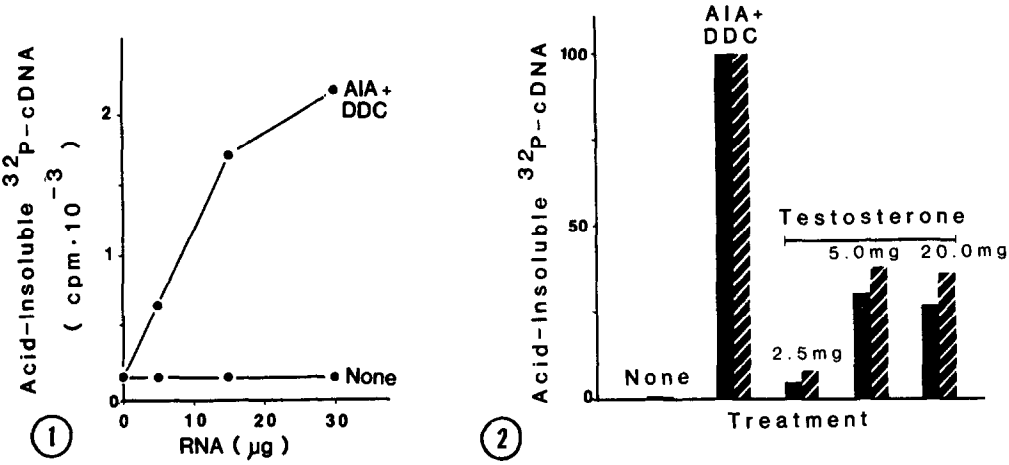

Fig. 1. Effect of RNA concentration on S_1 nuclease resistance of ALA synthase cDNA in solution hybridization reactions. A combination of the porphyrogenic agents AIA (2mg/embryo) and DDC (4mg/embryo) were dissolved in 0.1ml of dimethyl sulfoxide and injected into the fluid surrounding 18-day-old chick embryos. The embryos were then incubated for 14h at 38°C and liver RNA was isolated. Livers from 3-6 embryos were pooled for each determination. The values represent averages of cpm of acid-insoluble [^{32}P]-labeled cDNA remaining after digestion with S_1 nuclease in solution hybridization reactions. AIA + DDC refers to results of reactions with RNA from embryos treated with the porphyrogenic agents AIA and DDC. None refers to results of reactions obtained with RNA from embryos injected with the vehicle only.

Fig. 2. Effects of testosterone and of AIA + DDC on the level of ALA synthase-specific RNA as measured by solution hybridization. Total RNA was isolated from chick embryo liver as described in Experimental Procedures and analyzed by solution hybridization. The values represent averages of cpm of acid insoluble [^{32}]P-labeled cDNA remaining after S_1 nuclease digestion. The hybridizations were performed in triplicates with RNA isolated from two separate sets of embryos (solid and hatched). Livers from 3-6 embryos were pooled for each experimental condition. In each determination, 30µg of RNA was used. For each set of determinations, the values are expressed relative to the amount of [^{32}P]-cDNA obtained with reactions containing RNA from AIA + DDC treated embryos. None refers to results of reactions with RNA from embryos injected with the vehicle only. AIA + DDC refers to results of reactions with RNA from embryos treated with AIA (2mg/ml) and DDC (4mg/ml). Testosterone refers to results of reactions with RNA from embryos administered the indicated amounts of the steroid.

depended on the dose of testosterone, up to 5.0mg per embryo. The elevation in the concentration of RNA was greater when the two more potent inducers, AIA and DDC, were used. Since actinomycin D blocked the induction of ALA synthase activity by steroids (8), our data suggested that testosterone increased the rate of transcription of ALA synthase-specific RNA.

The activity of ALA synthase in liver appears to be regulated by free heme in the cells. Hemin inhibits the increase in the activity of the enzyme brought on by porphyrogenic agents (1). It is clear that hemin functions, in part, by inhibiting the transfer of the precursor form of ALA synthase from the cytoplasm into the mitochondria, resulting in the turnover of the precursor in the cytoplasm (9, 18, 19). However, a potential role by hemin in affecting the production of the protein by a different mechanism has not been tested directly. Using cDNA-RNA solution hybridization analyses, we examined the effect of hemin on the elevations in ALA synthase-specific RNA brought about by testosterone and by AIA. Since hemin was insoluble when injected into embryos, we resorted in those studies to using primary cultures of hepatocytes. Both AIA and testosterone caused significant elevations in the concentration of ALA synthase-specific RNA in the cells, and the addition of hemin into the culture medium of the hepatocytes inhibited the elevations (Fig. 3.). The most simple explanation of these data is that hemin inhibited the testosterone and the AIA induced production of ALA synthase mRNA at the level of transcription. However, the possibility that hemin might have also increased the rate of degradation of ALA synthase mRNA or of its nuclear precursors can not yet be dismissed. In any case, hemin clearly regulates the production of ALA synthase by affecting the concentration of the mRNA of the enzyme in hepatocytes treated with testosterone and with AIA.

The mechanisms through which porphyrogenic agents induce elevations in liver ALA synthase activity have not been determined. An agent may act by decreasing the free heme pool in hepatocytes, which may lead then to a derepression of the ALA synthase gene. On the other hand, an inducer may act directly on the gene to increase the rate of its transcription. AIA and DDC

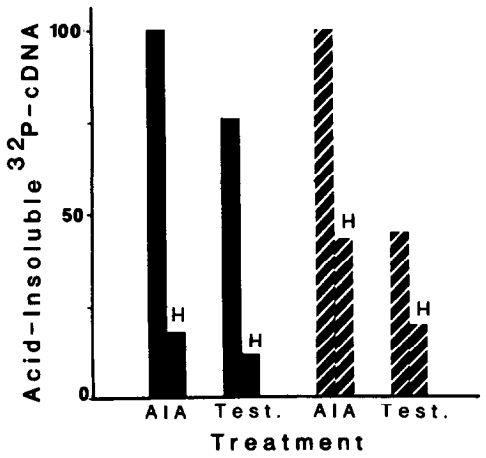

Fig. 3. Effect of hemin on ALA synthase-specific RNA levels in hepatocytes treated with testosterone or with AIA as measured by solution hybridization. Primary cultures of chick embryo hepatocytes were prepared and RNA was isolated as described in Experimental Procedures. For each experimental condition, RNA was isolated from eight 10cm culture plates (2-3mg protein/plate). The values represent average cpm of acid insoluble [^{32}P]-labeled cDNA obtained after digestion of samples with S_1 nuclease. The values are expressed relative to the amount of acid insoluble [^{32}P]-cDNA in reactions carried out with RNA from cultures treated with AIA. Control values, obtained with RNA isolated from hepatocytes treated with the vehicle alone, were at background. Values from the first preparation (solid) represent the averages of two independent determinations, where each determination was run in duplicates and with 40μg of RNA used in each hybridization. RNA was isolated 12h after the addition of testosterone or AIA into the medium, and 6h after the addition of 5μM hemin (H) as indicated. Values from the second preparation (hatched) represent the averages of duplicate determinations using 20μg of RNA in each hybridization reaction. RNA was isolated 6h after the addition of testosterone or AIA into the medium, and 6h after the addition of 2μM hemin (H) as indicated.

appear to induce the activity of the enzyme by lowering the concentration of heme in hepatocytes (20). In the case of certain steroid hormones, their presence in the medium of hepatocyte cultures has resulted in the accumulation of coproporphyrin (20). This suggests that steroids may inhibit coproporphyrinogen oxidase and thus block heme synthesis. Numerous steroid hormones also increase cytochrome P-450 activity in hepatocytes (5). Since newly-synthesized P-450 apoprotein must bind heme to form a functional cytochrome, elevation of the rate of production of the cytochrome may also result in the lowering of intracellular levels of heme. Thus, steroids may well bring about the derepression of the ALA synthase gene by lowering the concentration of free heme in hepatocytes.

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