Induction of liver apolipoprotein A-IV mRNA in porphyric mice

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ABSTRACT

We have isolated cDNA clones for mRNAs that are induced by porphyria from a mouse liver library. Of the three inducible clones isolated, we have identified one as being apolipoprotein A-IV (apo A-IV) by its extensive homology with a rat apolipoprotein A-IV cDNA sequence. The level of liver apo A-IV mRNA increases rapidly in response to either of two porphyrogenic drugs. When the ferrochelator-inhibited drug, 3,5-dicarboxy-1,4-dihydrocollidine (DDC) is used, a 6 and 28 fold induction of liver apo A-IV mRNA is observed in male and female mice, respectively. If the heme-destroying porphyrogenic drug, allylisopropylacetamide (AIA) is the inducing agent, liver apo A-IV mRNA levels increase 2-3 fold in both males and females. The level of apo A-IV mRNA reaches a maximum within 6-10 hr. after drug administration. Intestine apo A-IV mRNA levels do not change during either of these drug-induced porphyrias. RNA from acute-phase responsive liver or liver from mice treated with bilirubin, porphobilinogen, or protoporphyrin IX show no increase in apo A-IV mRNA. These results indicate that apo A-IV induction is tied to a disruption in porphyrin-heme biosynthesis but is not directly affected by several heme intermediates nor by the major heme degradation product, bilirubin.

INTRODUCTION

Porphyrias are diseases caused by disruptions in porphyrin-heme biosynthesis. They may be hereditary or chemically induced and they may affect either the liver or the red cell (for a review, see ref. 7). The effect of lowered heme levels on gene expression is of importance in defining and understanding the cellular response to porphyria. For example, it is known that certain porphyrogenic drugs increase the level of δ -aminolevulinic acid synthase (ALA-S) and its mRNA 10-20 fold²⁻⁴ (Buchberg and Kinniburgh, unpublished results). ALA-S is the first enzyme in the heme biosynthetic pathway. Elevated levels of aminolevulinic acid result in the accumulation of other heme intermediates, since the levels of other enzymes in the pathway do not increase. The ensuing hepatic porphyria results from the toxic effects of these intermediates. It seems likely that other changes in gene expression occur during the cellular response to porphyria. We therefore have isolated mouse liver cDNA clones

for mRNAs that increase during experimentally induced porphyria. We report here that one of these mRNAs is apolipoprotein A-IV (apo A-IV), a major serum apolipoprotein. Apo A-IV is involved in both exogenous lipid metabolism (chylomicron formation)^{6,7} and endogenous lipid metabolism (high density lipoprotein formation)⁸. The large increase in apolipoprotein A-IV mRNA that is independently elicited by two functionally different porphyrogenic drugs indicates that the apo A-IV protein may play an important role in the hepatic response to porphyria.

MATERIALS AND METHODS

Porphyrogenic drug injection

Male and female C3H mice were injected intraperitoneally with 0.1 ml to 0.25 ml of allylisopropylacetamide (AIA) (a gift from Hoffman-La Roche, Nutley, N.J.) or dicarbethoxy-1,4-dihydrocollidine (DDC) (Aldrich Chemical Co.), 130 mg each/Kg body weight. AIA was dissolved in 0.9% NaCl (w/v) and DDC was recrystallized twice from ethanol and dissolved in commercial olive oil. Mice injected with either saline, oil, or both were used as controls. See the legend to Table 1 for additional details. Other heme intermediates/catabolic products were purchased from Aldrich Chemical Co.

RNA preparation

Livers and small intestines were dissected and RNA was extracted by the guanidine hydrochloride procedure. RNA was quantitated by absorbance at 260 nm.

RNA electrophoresis and blotting

Ten g of total cell RNA was glyoxalated and electrophoresed through 1.2% agarose gel. RNA from the gel was blotted onto a nylon mesh, Zetabind (Cuno Inc.). The RNA was crosslinked to the Zetabind by exposing it to 254 nm UV-light for 3-5 min. using a hand-held light source. DNA was nick translated to a specific activity of $2-4 \times 10^6$ cpm/ μ g and 2×10^6 cpm/ml of hybridization buffer was used. Blots were then hybridized and washed by the method of Church and Gilbert⁹ and exposed to X-ray film (Kodak, XAG). Autoradiograms were quantitated by densitometric scanning. Dilutions of various RNA samples demonstrated that the hybridization signal is proportional to the amount of RNA present on the blot.

cDNA cloning and DNA sequencing

cDNA clones were prepared by a modification of the procedure of Okayama and Berg¹¹ (D. Dickinson and J. Toole, personal communication). Briefly, double-stranded cDNA was prepared by the above method and then C-tailed with terminal transferase¹². These cDNAs were inserted into Sst I cut and G-tailed pUC¹³. JW355 bacterial cells were transformed using the RbCl₂ procedure¹³.

DNA was sequenced using the chemical degradation method of Maxam and Gilbert¹⁴. Samples were run on 5% acrylamide-7 M urea gels of 0.4 mm x 120 cm x 35 cm.

Computer analysis

DNA sequence data was analyzed using the University of Minnesota and Cornell DNA programs on an Apple 2e microcomputer.

RESULTS

To isolate cDNA clones to mRNAs that increase their abundance during the genesis of experimental porphyria, we have performed the following experiments. First, mRNA that is enriched in sequences specific to porphyric liver was isolated. This was accomplished by hybridizing mRNA from porphyric mouse liver to cDNA-cellulose prepared with untreated mouse liver mRNA, isolating the unhybridized mRNA, and repeating the hybridization for a total of three rounds (Fig. 1). The final unhybridized mRNA fraction (which should lack most or all of the mRNAs common to untreated and porphyric liver) was used to prepare ³²P-labeled cDNA. This labeled cDNA was utilized to screen a mouse liver cDNA library that had been constructed using porphyric liver poly(A⁺) RNA and the vector, pUC 13¹¹ (J. Toole, unpublished) (Fig. 1). Using this procedure we have isolated six different clones.

To ensure that each of the isolated cDNA plasmids was homologous to an induced mRNA, Northern blots of RNA from untreated mouse liver and

Figure 1. Scheme for isolating cDNA clones for porphyria induced mRNAs. See Materials and Methods section for experimental details.

Figure 2. Dot matrix comparison of mouse apo A-IV cDNA sequence and rat apo A-IV cDNA sequence. Each dot represents a perfect six nucleotide match.

porphyric mouse liver were probed with each ³²P-labeled plasmid. Three cDNA plasmids showed significantly greater hybridization to RNA from porphyric mouse liver than from untreated mouse liver (data not shown). These were sequenced and compared to known nucleotide sequences. One clone was found to have significant homology to a rat apolipoprotein A-IV cDNA clone ¹⁵. Dot matrix analysis demonstrates that our mouse clone, p17, has homology to a rat apo A-IV sequence in both coding and 3' untranslated regions (Fig. 2). In addition, this cDNA clone hybridizes liver RNA that can be translated *in vitro* into a 46 Kd protein, the size of apo A-IV (data not shown). We conclude, therefore, that this clone is homologous to mouse liver apo A-IV mRNA.

To understand better the mechanism of apo A-IV mRNA induction in porphyric mouse liver, Northern blot hybridization was used to quantitate the increase in apo A-IV mRNA. Equal amounts of total liver RNA from control, AIA, DDC, or AIA plus DDC treated mice were glyoxylated, electrophoresed through an agarose gel, blotted onto a nylon mesh, Zetabind (Cuno Inc.), and probed with ³²P-labeled mouse apo A-IV cDNA ¹⁰. A ³²P-labeled mouse alcohol dehydrogenase cDNA was simultaneously hybridized to the blot as an internal control. The ADH signal was useful in correcting for cumulative differences in RNA transfer efficiency, hybridization efficiency, and other experimental variables. The results show that DDC alone or in combination with AIA produces a large increase in liver apo A-IV mRNA of both male and female mice (Fig. 3A). AIA alone

Table 1

Induction of Liver Apo A-IV mRNA with Porphyrogenic Drugs				
	Levels of mRNA (arbitrary units)			
	Apo A-IV	ADH	Apo A-IV (corrected)	Apo-IV fold induction
♀ control	2.6	4.6	0.6	-
♀ DDC	25.0	1.5	16.7	28
♀ DDC+AIA	14.1	1.2	11.8	20
♀ control	7.0	2.7	2.5	-
♀ AIA	22.4	4.2	5.3	2
♂ control	1.8	1.2	1.5	-
♂ DDC	14.0	1.2	11.6	8
♂ DDC+AIA	10.0	1.2	8.3	6

The following protocol was used for this experiment. Food was withdrawn 14 hr. prior to injection. Animals were given two injections of either DDC, AIA, or both (at 130 mg each/Kg body weight) at 0 hr. and 10 hr. Controls were injected with either olive oil (DDC controls) or 0.9% NaCl, w/v (AIA controls). Mice were killed at 24 hr. post-injection and RNA was prepared as described in Materials and Methods.

1. Data normalized to ADH internal control
2. DDC, dicarbethoxy-1,4 dihydrocollidine
3. AIA, allyisopropylacetamide

produces a smaller response than DDC alone in female mice (Fig. 3B). A similar increase in liver apo A-IV mRNA is seen in AIA-treated male mice (data not shown). The variably observed, faint band between the ADH and apo A-IV mRNAs is due to nonspecific hybridization of one or both probes to 18S rRNA. The levels of apo A-IV mRNA in the above samples were quantitated by densitometric scanning of the autoradiograms. That this method yields quantitative results was demonstrated in the following way. RNA samples were serially diluted and the level of apo A-IV mRNA was quantitated as described above. The hybridization of labeled probe was found to be directly proportional to the amount of RNA (data not shown). The ADH mRNA was similarly quantitated and used to correct for small variations in the hybridization among samples, since its level did not vary greatly under any experimental condition. The data demonstrate that DDC induces apo A-IV mRNA levels 8 and 28 fold in males and females,

Figure 3. Northern blot analysis of liver apo A-IV and ADH mRNA levels. Ten μg of glyoxylated RNA were electrophoresed through a 1.2% agarose gel, blotted onto Zetabind, and probed with 2×10^6 cpm/ml of apo A-IV cDNA and 2×10^6 cpm/ml ADH cDNA probe, labeled to specific activities of 3×10^8 cpm/ μg each. A. C3H mouse liver RNA from, 1. female, oil injected control; 2. female, DDC injected; 3. male, oil injected control; 4. male, DDC injected; 5. female, DDC and AIA injected; 6. male, DDC and AIA injected. B. Mouse liver RNA from, 1. female, saline injected control, 2. female; AIA injected. DDC and AIA were both injected at 130 mg/Kg body weight.

respectively (Table 1). AIA induces apo A-IV mRNA levels approximately 2 fold in females (Table 1) and 2-3 fold in males (data not shown). When both drugs are injected, the induction of apo A-IV mRNA is similar to that found for DDC alone, 6 and 20 fold for males and females, respectively (Table 1). Although the absolute basal (uninduced) levels and induced

Figure 4. Time course of apo A-IV mRNA induction with porphyrogenic drugs. Female mouse liver RNA was analyzed by Northern blot/apo A-IV probe hybridization. Autoradiographs (upper right-hand corners) were scanned to quantitate apo A-IV mRNA levels. The absorbance (apo A-IV mRNA quantity) is plotted versus time after injection. Top, DDC injected; bottom, AIA injected.

levels of apo A-IV each vary by 50-100% in the four experiments performed to date, females consistently have a greater induction of apo A-IV mRNA with DDC. In these experiments, mice were rendered porphyric by injection of drugs at 0 hr. and 10 hr. and liver RNA was prepared 24 hr. post-injection. This double-injection protocol causes a larger induction of apo A-IV mRNA than a single-injection protocol when DDC or AIA is administered (see below). The reasons for the sex differences and for the injection protocol differences are not known at present.

To investigate possible mechanisms of apo A-IV mRNA induction, we studied the kinetics of the mRNA increase. Either AIA or DDC was injected singly, and liver RNA was prepared at various times post-injection. The level of apo A-IV mRNA was assayed by Northern blot hybridization and quantitated as in Figure 3 and Table 1. Both the AIA and the DDC treated

mice showed similar, relatively rapid increases in liver apo A-IV mRNA (Fig. 4). Maximal levels of apo A-IV mRNA were attained by 10 hours post-injection. mRNA levels decreased slightly between 10 and 24 hours, and again between 24 and 48 hours post-injection (Fig. 4). This response is relatively rapid when compared to other mRNA induction, such as DMSO induction of globin mRNA in Friend cells, which may take 20-36 hrs. for maximal RNA levels to be reached. Both drugs showed a similar level of apo A-IV mRNA induction (2 fold). As stated above, the large 10-20 fold DDC-induced increase and the 6-8 fold AIA-induced increase in apo A-IV mRNA is observed only when multiple injections are performed. Why the multiple injection protocol (obtained from ref. 2) gives this large difference in inducible apo A-IV mRNA is not known. Dose-response experiments are currently underway.

Several further experiments were performed to understand whether apo A-IV mRNA levels increase due to heme biosynthetic pathway intermediates, heme degradation products, or a general liver toxicity. The heme-porphyrin intermediates uroporphobilinogen and protoporphyrin IX were injected into female mice in a wide range of concentrations. Bilirubin, the terminal heme oxidation product, was also injected in varying amounts, and apo A-IV mRNA was assayed as described above. No induction of apo A-IV mRNA was observed with any of these compounds (data not shown). It is possible that these drugs were not delivered to the liver (this seems unlikely in the case of bilirubin, since it is targeted to the liver for excretion into the bile). We are further investigating the possibility that the primary inducing agent is a heme intermediate. In addition, liver RNA from mice treated with turpentine, to elicit the liver acute-phase response¹⁶, was analyzed for apo A-IV mRNA. No increase in apo A-IV mRNA was observed in acute-phase liver (data not shown).

The apo A-IV protein is found in the intestine, where it participates in chylomicron formation, and the liver, where it presumably is synthesized and secreted⁶. We reasoned that if apo A-IV mRNA induction was specific for the hepatic response to porphyria, then intestine apo A-IV mRNA would not increase in porphyric mice. On the other hand, if the porphyrogenic drugs induce a general humoral response, then intestine apo A-IV mRNA levels might increase in porphyric mice. To answer this question, intestine RNA was prepared from both male and female DDC-induced porphyric mice. It can be seen that there is no significant difference in intestinal apo A-IV mRNA levels among untreated or DDC-treated male and female mice (Fig. 5). The liver apo A-IV mRNA levels of these mice are those shown in Figure 3. ADH mRNA could not be detected in any intestine RNA preparation, as expected from known ADH tissue distribution.

Figure 5. Intestine apo A-IV mRNA levels in control and porphyric mice. Ten micrograms of mouse small intestine RNA was electrophoresed, blotted, and hybridized as described in Fig. 3. Lane 1, female, oil injected control; lane 2, male oil injected control; lane 3, female DDC treated; lane 4, male DDC treated.

DISCUSSION

We have cloned several mRNAs that increase in abundance in response to experimentally induced porphyria. One of these cDNA clones was identified as apolipoprotein A-IV by its homology to rat apo A-IV and its ability to hybridize mRNA that can be translated into a protein of appropriate molecular weight (Fig. 2, data not shown). Female mice show a somewhat greater induction than males when porphyria is induced with either of two drugs singly or in combination (Fig. 3, Table 1). When DDC, an inhibitor of the last enzyme in the heme pathway, ferrochelatase, is used as the porphyrogenic agent, an 8-28 fold increase is seen in apo A-IV mRNA levels (Fig. 3, Table 1). If AIA, an *in situ* heme oxidizing agent, is employed to induce porphyria, then a 2-3 fold increase in apo A-IV mRNA is observed (Fig. 3, Table 1, data not shown). These differences probably reflect a true drug-specific bias, since other of our porphyria-inducible cDNA clones have mRNAs that show the opposite drug preference (Buchberg and Kinniburgh, in preparation). The kinetics of apo A-IV mRNA induction are similar with each drug. Apo A-IV mRNA increased by 4-6 hr. and reached maximal levels by 10 hr. post-injection (Fig. 4). The apo A-IV mRNA levels then decreased slowly between 10 hr. and 48 hr. post-injection to 50% of their maximal level (Fig. 4). This relatively rapid increase in apo A-IV mRNA suggests that porphyria either 1.) stabilizes apo A-IV transcripts (in this case the $t_{1/2}$ of the non-porphyric apo A-IV RNA must be 10 hr. or

the kinetics of induction would appear slower), 2.) increases the transcription of the apo A-IV gene, or 3.) involves a combination of increased RNA stability and increased transcription. Experiments are currently underway to decide which of these mechanisms contributes to the rapid increase in apo A-IV mRNA.

The physiologic basis of the large increase in apo A-IV mRNA is at present not known. We believe, however, that several characteristics of this response point toward a significant physiological role. 1.) The apo A-IV response to porphyrogenic drugs is limited to the target tissue; liver apo A-IV mRNA levels increase but not intestine apo A-IV mRNA (Figs. 3, 5). 2.) The response is rapid; liver apo A-IV mRNA reaches maximal levels between 6-10 hr. after injection of the porphyrogenic drug (Fig. 4). 3.) Two drugs that induce porphyria in fundamentally different ways each produce an increase in liver apo A-IV mRNA abundance (Fig. 3). 4.) Other inducers of hepatic gene expression, such as turpentine's induction of the acute-phase response, do not increase apo A-IV mRNA levels (data not shown). Taken together, these data suggest an important physiological link between porphyria and apo A-IV gene expression. Two major physiologic roles for apo A-IV mRNA induction during porphyria are suggested by our data: 1.) Apo A-IV gene expression is increased as an adaptive response to the porphyria, e.g., apo A-IV acts as a detoxificant, or 2.) A rapid imbalance of hepatic lipid metabolism occurs during porphyria. The subsequent induction of apo A-IV would be, therefore, a response to its function in lipid metabolism. Further experimentation will be needed to define apo A-IV's role in porphyria.

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