ANTAGONISTIC EFFECT OF FePP ON THE ETHANOL MEDIATED INDUCTION OF HEPATIC, RENAL AND SPLENIC δ -AMINO LEVULINIC ACID SYNTHASE ACTIVITY *IN VIVO* IN RATS

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ABSTRACT

In the current study, we report perturbations in hepatic, renal and splenic heme synthesis at the level of the rate limiting enzyme, δ -amino levulinic acid synthase (ALA-S) on ethanol administration independently and in conjunction with iron-protoporphyrin (FePP) in male Wistar rats. Excessive ethanol administration (5 ml/kg bw) resulted in a significant induction of hepatic, splenic and renal ALA-S activity. Simultaneous administration of FePP (50 μ mol/kg bw) reverted the observed induction response to a sharp decline. The features of the action of ethanol and FePP together *in vivo*, i.e. a substantial inhibition of ALA-S is suggestive of the beneficial effects of this formulation in acute attacks of porphyria.

KEY WORDS: Ethanol, Heme biosynthesis, delta-amino levulinic acid synthase (ALA-S), Iron Protoporphyrin (FePP)

INTRODUCTION

The rate limiting enzyme of heme biosynthesis, δ -amino levulinic acid synthase (ALA-S) (EC 2.3.1.37) is the first enzyme which catalyses the condensation of glycine and succinyl CoA to form δ -amino levulinic acid. ALA-S has a very short half life (~60 minutes) and thus, measurement of its activity essentially reflects the rate of enzyme production (1,2). There is now ample evidence that heme biosynthesis in the liver of animals is subject to feed back regulation by heme, the end product of the pathway (2-4). Specifically, heme negatively regulates both the synthesis (5,6) and intracellular transfer of the enzyme (7) causing a reduction of the ALA-S enzyme level in liver mitochondria. This feed back regulation by heme is an attractive mechanistic control circuit in the animal system, since heme exerts its regulatory effect through multiple feed back pathway to reduce the amount of regulatory enzyme in the organelles where the

enzyme functions. ALA-S is located in the mitochondrial matrix (8) but is synthesized on free cytoplasmic ribosomes as a larger precursor form (9). This mitochondrial enzyme has been shown to be regulated by a variety of factors, including nutritional status, hormones, porphyrinogenic agents and other xenobiotics (10). The enzyme induction is associated with acute intermittent porphyria (AIP), an inherited liver disorder in humans characterised by elevations in the activity of ALA-S and an overproduction of porphyrins (11).

The regulatory effects of heme on heme biosynthesis are multiple i.e. inhibition of ALA-S synthesis at both transcriptional and translational steps, inhibition of the enzyme transfer from cytosol into mitochondria and inhibition of its catalytic activity (12). To our knowledge, ALA-S is the only example for which the occurrence of regulation of intracellular translocation is clearly demonstrated. We may assume that this inhibition represents a new mechanism of feedback regulation of metabolism in the sense that this is realized by

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regulation of the intracellular translocation of the key enzyme of the metabolic pathway.

Okuno et al (13) reported that ethanol consumption in rats increases the activity of hepatic ALA-S significantly and that of the heme oxygenase only slightly. The foregoing study is an attempt to investigate the intercalative effect of co-administration of alcohol and FePP on the hepatic, splenic and renal ALA-S activity in the Male Wistar Rats.

Alcohol has a variety of pathologic effects on hematopoiesis. It directly damages erythroid precursors, thereby contributing to macrocytosis and the anemic state of chronic alcoholics. Ethanol induces siderblastic anemia, perhaps by direct interference with heme synthesis. Further, chronic injection of alcohol can lead to various types of hemolytic anemia caused by alterations in the erythrocyte membrane lipids which occur in association with alcoholic liver disease (14,15).

Recent reports indicate that ethanol administration results in hepatic oxidative stress observed in rats. The ethanol-inducible cytochrome P450 2E1 plays a key role in its generation, favoured itself by an increase in the 'redox active' fraction of intracellular non heme iron (16). Interaction of alcohol and drugs in the liver appears to involve common microsomal oxidative enzymes which utilize cytochrome P-450. Since alcohol augments the toxicity of a variety of drugs, the regulation of the P-450 hemoprotein, a primary component in hepatic drug metabolizing systems, may play a vital role in this phenomenon (17). Since liver is predominant in the metabolism of ingested ethanol, we converged upon evaluating the effect of ethanol administration independently as well as in conjunction with FePP on the heme biosynthetic enzyme viz., ALA-S.

To explore the importance of combination dosages, we undertook the investigation of excessive ethanol (5 ml/kg bw) administration in association with FePP (50 μ mol/kg bw) on the hepatic, splenic and renal ALA-S activity in male Wistar rats.

MATERIALS AND METHODS

Chemicals

FePP was purchased from Porphyrin Products, Logan, Utah, USA. The compound was analysed for purity by thin layer chromatography on precoated Silica Gel G plates (Merck) using the following system: water, n-propanol and pyridine 5.5: 0.1: 0.4 by volume. All other chemicals and reagents were of highest analytical grades available commercially.

Experimental animals

Male Wistar rats of weight range 150-200g from our laboratory maintained colony were used as experimental models in the investigation. Only healthy animals were taken in individual cages having raised wire mesh floors. The animals were kept on fasting for 24 hours but had free access to water. After 24 hours, the animals were divided into four groups with six animals per group.

Animal treatment

Group I : Animals of this group were treated as control and were administered equivalent amount of saline subcutaneously. **Group II :** 5 ml/kg bw of ethanol was given subcutaneously to animals in this group. **Group III :** Animals in this group were administered 50 μ mol/kg bw of FePP subcutaneously. **Group IV :** Animals in this group were given 5 ml/kg bw of ethanol along with 50 μ mol/kg bw of FePP subcutaneously.

Solution of the metalloporphyrin was prepared in diffused light by dissolving the compound in 1 ml of 0.2 N NaOH, adjusting the pH to 7.4 with 1M HCl, and diluting the solution to a final volume with 0.9% NaCl. This metalloporphyrin solution was administered within 10 minutes of preparation.

Isolation of mitochondrial fraction

After 24 hours, the animals were sacrificed using ether for anesthesia. Liver, spleen and kidney

were quickly dissected out, immersed in ice-cold saline, cleaned, wiped and dried, and weighed. The tissues were homogenized and the fractionation of tissue homogenates of the control and experimental rats in subcellular components was carried out in 0.25 M sucrose solution according to the method of Hogeboom (18) and Umbreit et al. (1955) (19).

ALA-Synthase assay: ALA-Synthase activity was assayed colorimetrically by the method of Sassa et. al. (20) as follows: homogenates were centrifuged at $8,000 \times g$ for 15 min at 4°C and supernatants were discarded. Pellets were resuspended to restore the original volume with a reaction mixture containing in micromoles/ml, of glycine(75), citrate(75), tris buffer, pH 7.4 (37.5), phosphate buffer, pH 7.4 (37.5), MgCl_2 (15), EDTA (7.5), and pyridoxal 5'-phosphate (2.2). Triplicates of 300 μl aliquots were incubated at 37°C for 60 min in a metabolic shaker at 60 oscillations/min. The reaction was terminated by the addition of 100 μl of ice cold 10% trichloroacetic acid. The reaction mixture was then centrifuged at $1000 \times g$ for 5 min, and 300 μl of the supernatant was transferred to another tube and mixed with 150 μl of 10% 2,4-pentanedione in 1M sodium acetate. The mixture was then heated at 80°C for 10 min to convert ALA to 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole. After the tubes were cooled, the pH of the mixture was adjusted to 7 with 50 μl of 0.5 M Na_2HPO_4 , 1N NaOH (1:3 v/v). The mixture was vigorously mixed with 750 μl of CH_2Cl_2 . This procedure removes 92% of the aminoacetone pyrrole formed and less than 9% of 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole (21). The aqueous phase was then removed and mixed with an equal volume of the modified Ehrlich reagent (21). A difference spectrum between the color salt of the incubated sample and the unincubated control was obtained in the wavelength range 650 nm to 500 nm. The concentration of ALA formed was determined based on the difference in absorbance at 553 nm and 650 nm using an extinction coefficient of $58 \text{ mM}^{-1}\text{cm}^{-1}$ (22)

Protein estimation

Protein estimation of the mitochondrial fraction of the tissues was done according to the method of Lowry et. al. (23) using bovine serum albumin as standard.

Statistical analysis

The difference between control and treated animals were assessed by the Analysis of Variance (F-test). Value of 'F' at 0.05 level of significance was calculated.

RESULTS

Effect on ALA-S

In our study on the effect of FePP in concurrence with ethanol, our results revealed that ethanol administration caused a significant stimulation of hepatic ALA-S activity by ~1.5 fold. We observed an enhancement of ALA-S activity in the spleen and kidney by ~2 fold and ~90% respectively. FePP administration evoked a decline in hepatic ALA-S activity by ~25%. An inhibition of ALA-S activity by ~30% and ~40% was observed in the spleen and kidney respectively on FePP administration. Simultaneous administration of FePP and ethanol declined the hepatic, splenic and renal ALA-S activity by ~90%, ~75% and ~85% respectively (Fig.1).

Effect on total mitochondrial protein

Ethanol administration decreased hepatic mitochondrial protein content by ~30% as observed after 24 hours. The protein levels showed a diminution on ethanol administration in the spleen and kidney by ~35% and ~20% respectively as compared to control levels. FePP administration decreased the hepatic mitochondrial protein concentration by ~35%. FePP produced a decrease in protein levels in the spleen and kidney by ~45% and ~60% respectively. FePP in conjunction with ethanol elicited a decline in mitochondrial protein levels by ~50%, ~60% and ~75% in the liver, spleen and kidney respectively (Fig. 2).

Fig. 1. Effect of ethanol and FePP on ALA-S activity in the liver, spleen and kidney. Adult male Wistar rats were administered (5 ml /kg bw) ethanol, and (50 μ mol/kg bw) FePP subcutaneously. Data were analysed by "f" test, and are presented as the means $\pm$ S.E. of six determinations.

Fig. 2. Effect of ethanol and FePP on total mitochondrial protein concentration in the liver, spleen and kidney. Adult male Wistar rats were administered (5 ml /kg bw) ethanol, and (50 μ mol/kg bw) FePP subcutaneously. Data were analysed by "f" test, and are presented as the means $\pm$ S.E. of six determinations.

DISCUSSION

Despite the inherent difficulties in interpreting *in vivo* data on the effects of alcohol, literature survey underscores that ethanol itself alters the metabolism of porphyrins and in doing so directly influences heme and hemoglobin synthesis. The mechanism of ethanol-induced iron overload remains elusive but, through observing the effects of a rechallenge with iron, the relationship of iron and alcohol to porphyria cutanea tarda are being more clearly defined. The control mechanisms of porphyrin synthesis by endogenous factors may be part of a more general red blood regulatory apparatus (24). It is believed that heme regulates its own synthesis by feed back repression of ALA-synthase, the rate limiting enzyme in the heme biosynthetic pathway. The observation elicited on FePP administration on the rate limiting enzyme of the heme biosynthesis viz. ALA-S, can be plausibly explained on the basis of initial repression by heme of the synthesis of ALA-S, followed by de-repression and an undamped overshoot in ALA-S after excess of heme was catabolised. The simultaneous administration of ethanol along with FePP disturbs this cycle and leads to a significant decline of ALA-S activity.

Our results revealed that ethanol stimulates the activity of ALA-S in the liver, spleen and kidney but the combined effect of ethanol and FePP results in a rapid drop in intracellular heme concentration i.e. co-administration of both elicits a substantial decline in the activity. It has been suggested that there is a competition for intracellular heme for the synthesis of various hemoproteins, including the microsomal cytochromes P-450 and b_5 , the mitochondrial cytochromes, catalase and tryptophan pyrrolase, and that the remaining uncommitted to apoprotein serves either to repress the synthesis of ALA-S (and thus decrease net heme synthesis) or is catabolised to bile pigment via heme oxygenase (HMOX). The observed inhibitory effect of FePP may be due either to its binding or interaction specifically with the precursor form of the enzyme so as to interfere with the interaction of the precursor

protein with the component(s) of the transport machinery or to a specific interaction of ethanol and/or iron-protoporphyrin with the peptide chains of the enzyme necessary for inhibition. Xenobiotics clearly regulate the production of ALA-S by affecting the concentration of the mRNA of the enzyme in the tissues of animals, treated with ethanol and ethanol along with FePP in this case.

The blocking action by co-administration of FePP itself is striking and consistent with other data which demonstrates that certain metalloporphyrins can significantly modify the effects produced in biological systems.

We plausibly may also put forth that the observed effect may be, is not caused by alcohol itself but either by its metabolites formed due to the action of FePP or by the changes of the liver metabolism caused by disturbances in the NAD/NADH ratio.

An important concept which has emerged from these studies is that ALA-S activity is regulated in a tissue specific manner. Viewed as a whole, the studies demonstrate that the regulation of ALA-S differs in each tissue studied.

It is worth exploring whether the combination dosage can be a therapeutically useful formulation in acute attacks of porphyria, specifically acute intermittent porphyria characterised by a defect in the specific enzyme, ALA-S of the heme biosynthetic pathway.

Acknowledgements

The author RC thankfully acknowledges the University Grants Commission, New Delhi for Career Award and Research Scientist Award, and the Rockefeller Foundation, USA for the Biotechnology Career Award. Another author CR acknowledges the facilities rendered by Dr. B.R. Ambedkar Center for Biomedical Research during the SURP (Summer Undergraduate Research Program) for the execution of this work.

REFERENCES

1. Granick, S. and Urata, G. (1966) The induction in vitro of the synthesis of delta-aminolevulinic acid synthetase in chemical porphyria: a response to certain drugs, sex hormones, and foreign chemicals. *J Biol. Chem.* 241(6), 1359-1375.
2. Yamamoto, M., Kure, S., Engel, J.D. and Hiraga, K. (1988) Structure, turnover, and heme-mediated suppression of the level of mRNA encoding rat liver delta-aminolevulinate synthase. *J. Biol. Chem.* 263(31), 15973-15979.
3. Kikuchi, G. and Hayashi, N. (1982) In: Structure, dynamics and biogenesis of biomembranes. Eds. Sato, R. and Omishi, S., Japan Scientific Societies Press, Tokyo. 131-156.
4. Kappas, A., Sassa, A. and Anderson, K.E. (1983) In: The metabolic basis of inherited disease. Eds. Stanbury, J.B., Wyngarden, J.B., Fredrickson, D.S., Goldstein, J.L. and Brown, M.S. McGraw Hill Book Co., New York. 1302-1384.
5. Maines, M.D. (1997) The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharmacol. Toxicol.* 37, 517-54.
6. Yamamoto, M., Hayashi, N. and Kikuchi, G. (1983) Translational inhibition by heme of the synthesis of hepatic delta-aminolevulinate synthase in a cell-free system. *Biochim. Biophys. Res. Commun.* 115(1), 225-231.
7. Hayashi, N., Watanabe, N., and Kikuchi, G. (1983) Inhibition by hemin of *in vitro* translocation of chicken liver delta-aminolevulinate synthase into mitochondria. *Biochim. Biophys. Res. Commun.* 115(2), 700-706.
8. Sassa, S., and Kappas, A. (1981) Genetic, Metabolic and Biochemical aspects of the porphyrias. In: *Adv. Hum. Genet.*, Eds. Harris, H. and Hirschhorn. Plenum Press, New York. vol. 11, p 121.
9. Whiting, M.J. (1976). Synthesis of delta-aminolaevulinate synthase by isolated liver polyribosomes. *Biochem. J.* 158(2), 391-400.
10. Condie, L.W., Baron, J. and Tephly, T.R. (1976) Studies on adrenal delta-aminolevulinic acid synthetase. *Arch. Biochim. Biophys.* 172(1), 123-129.
11. Bradlow, H.L., Gillette, P.N., Gallagher, T.F. and Kappas, A. (1973) Studies in porphyria. II. Evidence for a deficiency of steroid delta-4-5-alpha-reductase activity in acute intermittent porphyria. *J. Exp. Med.* 138(4), 754-763.
12. Bissell, D. M. and Hammaker, L.E. (1976) Cytochrome p-450 heme and the regulation of delta-aminolevulinic acid synthetase in the liver. *Arch. Biochem. Biophys.* 176(1), 103-112.
13. Scharf, R.E. and Aul, C. (1988) Alcohol-induced disorders of the hematopoietic system. *Z Gastroenterol.* 26, (Suppl. 3), 75-83.
14. Waller, H.D. and Benohr, H.C. (1978) Alcohol-related disturbances in haematopoiesis. *Klin Wochenschr.* 56(6), 259-265.
15. Nordmann, R. and Rouach, H. (1996). Alcohol and free radicals: from basic research to clinical prospects. *Ann. Gastroenterol. Hepatol. (Paris).* 32(3), 128-133.
16. Lane, S.E. and Stewart, M.E. (1983) Alcohol-mediated effects on the level of cytochrome P-450 and heme biosynthesis in cultured rat hepatocytes. *Biochim. Biophys. Acta.* 755(3), 313-317.
17. Hogeboom, G.H. (1995), In: *Methods in Enzymology*, Eds. Colowick S.P. and Kapiian, N.O. Academic Press Inc., New York. vol. 1, p. 16.
18. Umbeit, W.W., Burries, R.H., and Stauffer, S.F., (1957) Burgess Publishing Co., Minneapolis. 111rd edn.

19. Sassa, S., Kappas, A., Berstein, S.E. and Alvares, A.P. (1979) Heme biosynthesis and drug metabolism in mice with hereditary hemolytic anemia. Heme oxygenase induction as an adaptive response for maintaining cytochrome P-450 in chronic hemolysis. J. Biol. Chem. 10, 254(3), 729-735.
20. Lowry, O.H., Rosenbraugh, N.I., Farr, A.L. and Randell, R.J. (1951) Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193, 265-274.
21. Granick, S. (1966) The induction *in vitro* of the synthesis of delta-aminolevulinic acid synthetase in chemical porphyria: a response to certain drugs, sex hormones, and foreign chemicals. J. Biol. Chem. 241(6), 1359-1375.
22. Ostroff, J.W. (1981) The effect of ethanol on heme and porphyrin synthesis. Alcohol Clin. Exp. Res. 5(1), 125-130.
23. Held, H. (1977) Effect of alcohol on the heme and porphyrin synthesis interaction with phenobarbital and pyrazole. Digestion. 15(2), 136-146.