Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 306-312

THE EFFECT OF METALLOPORPHYRINS AND HEME LIPOSOMES ON δ-AMINOLEVULINATE SYNTHASE ACTIVITY IN RAT LIVER

John B. Cannon*[1], Fu-Shin Kuo[1], Fariba Vatandoust[1], H. Heng Liem[2] and Ursula Muller-Eberhard[2]

[1]Department of Chemistry
Cleveland State University, Cleveland, Ohio

[2]Division of Pediatric Hematology
Cornell University Medical College, New York, New York

Received May 29, 1985

SUMMARY: Heme administration causes inhibition of δ-aminolevulinate synthase (ALAS), best tested in the allylisopropylacetamide (AIA)-treated rat, a model for hepatic porphyrias. Because heme suspended in aqueous media (for injection) is unstable and has adverse effects on coagulation, alternate therapeutic modalities are being explored. The present study tries to answer two questions: 1) are any heme analogs as effective inhibitors of ALAS as heme is; and 2) does heme administration in the form of liposomes increase its effectiveness? None of the liposome compositions tested, even if containing lactosylceramide for preferential hepatocyte uptake, was more effective in inhibiting AIA-induced ALAS activity than heme in buffer. As for the function of the heme analogs, although deuteroheme and heme dimethyl ester proved ineffective, mesoheme and cobalt protoporphyrin were nearly as effective as heme itself, indicating that both hydrophobic side chains in positions 2 and 4 and free propionate groups at 6 and 7 are essential for ALAS inhibition, as is the presence of a central cobalt or iron atom.

δ-Aminolevulinate synthase (ALAS, EC 2.3.1.37) is the first and rate-limiting enzyme in the biosynthesis of heme (1) and heme has been shown to be a feedback repressor of liver mitochondrial ALAS (2). Hepatic ALAS levels are elevated in most forms of inducible porphyrias, which are caused by partial enzymatic defects in the heme biosynthetic pathway (3). Injection of heme effectively blocks the induction of ALAS in an animal model of the porphyrias,

* - Address correspondence to this author, present address:
Agricultural Research Division/American Cyanamid Company
P.O. Box 400 Princeton, NJ 08540

Abbreviations:

AIA, allylisopropylacetamide; ALA, δ-aminolevulinate; ALAS, δ-aminolevulinate synthase; chol, cholesterol; CP, dicetyl phosphate; FeTPPS, iron (III) tetraphenylporphyrin sulfonate; LC, lactosylceramide (DL-dihydrolactocerebroside, N-stearoyl); PBS, phosphate buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4); PC, phosphatidyl choline; SA, stearylamine. 'Heme' refers to iron protoporphyrin IX regardless of oxidation state.

0006-291X/85 $1.50

the allylisopropylacetamide-treated rat (4), and brings about biochemical and clinical improvements in exacerbations of the inducible porphyrias (5-7).

Oxidized heme, ferriprotoporphyrin, readily aggregates and is somewhat unstable in aqueous media (8,9), which may account for the adverse effects during treatment of porphyrias, in particular its effects on coagulation (10-13). Several porphyrins and metalloporphyrins other than heme are more soluble and stable and may therefore be preferable therapeutic modalities. An alternate approach to heme delivery could be its incorporation into liposomes, minimizing the interactions of heme with blood constituents and possibly raising the therapeutic index (ALAS inhibition/heme administered).

In this work we explored both of these possibilities in the AIA-treated rat. We first administered several porphyrins and metalloporphyrins in aqueous medium to determine if any of the more soluble or stable porphyrins or metalloporphyrins tested might be more effective than heme. We found mesoheme and Co-protoporphyrin to inhibit hepatic ALAS to approximately the same extent as heme. Heme was then partitioned into liposomes of various compositions as previously described (14). Heme incorporated into liposomes was no more effective than heme suspended in buffer, even if the liposomes contained glycolipid for preferential uptake by parenchymal liver cells.

MATERIALS AND METHODS

Materials: Hemin chloride, protoporphyrin IX dimethyl ester, and all lipids were purchased from Sigma (St. Louis, MO). Protoporphyrin IX and LC were purchased from Calbiochem (La Jolla, CA); all other porphyrins were purchased from Porphyrin Products (Logan, UT). [1,2-^{3}H]cholesterol (47 mCi/mmol, in benzene solution) was purchased from New England Nuclear (Boston, MA). AIA was donated by Hoffmann-La Roche (Nutley, NJ). FeTPPS was a gift from Dr. R. F. Pasternack (Department of Chemistry, Swarthmore College, Swarthmore PA 19081). Hemin dimethyl ester and mesoheme were prepared by insertion of iron into protoporphyrin dimethyl ester and mesoporphyrin, respectively (15a). Deuteroheme was synthesized from protoheme by reaction with resorcinol (15b). [^{14}C]Heme was prepared by incubation of [4-^{14}C]ALA (45 mCi/mmole; Research Products International, Mount Prospect, IL) with reticulocytes from chickens treated with phenylhydrazine, and subsequent crystallization of the heme by the method of Vogel (16). Porphyrin and metalloporphyrin solutions were prepared fresh daily and used within 1 h; they were dissolved in 0.1 M NaOH, adjusted to pH 7.5 with 0.1 M sodium phosphate buffer, and diluted with PBS. Sonicated heme liposomes were prepared as previously described (14), except that the ratio of heme to lipid was 1:10 (w/w) and the final gel filtration step was omitted. Heme, mesoheme and deuteroheme concentrations were determined by the pyridine hemochrome method (15c). Heme dimethyl ester was incorporated into liposomes by the addition of the metalloporphyrin in

methylene chloride/ether 4:1 solution (0.5 ml) to liposomes in buffer (2 ml), followed by evaporation of the organic solvent and sonication for 5 min.

Male Spraque-Dawley rats, ca. 150 g each, were purchased from Hilltop Lab Animals (Scottdale, PA). Animals were starved for 24 h before sacrifice and injected subcutaneously with 20 mg/ml AIA (400 mg/kg) in physiological saline 16-18 h before sacrifice. Treatment solutions (porphyrins and metalloporphyrins) were injected into the tail vein. Animals were killed by decapitation and the livers perfused *in situ* with 100 ml ice-cold PBS. The livers of 2-3 identically treated animals were pooled and homogenized in 3 volumes of cold 0.1 M sodium phosphate, pH 7.4. The ALAS activity was determined in the 9000 g pellet by the method of Sassa et al. (17). For radioactivity determinations, the homogenate was digested by Scintigest (Fisher Scientific) and counted in Scintiverse cocktail (Fisher Scientific) in a Beckman LS 7500 scintillation counter.

RESULTS AND DISCUSSION

In preliminary experiments we determined the optimal time and dosage schedule for the inhibitory effect of heme on ALAS activity in the AIA-treated rat. One hour after heme injection maximal inhibition was reached, as Yamamoto had reported (4). The ALAS activity, which had been raised from a control value of 16 ± 4 (in 16 normal rats) to 85 ± 9 nmol/g liver/h (in 27 AIA-treated rats) (Table I), was reduced to approximately half that value when

Table I: Effect of porphyrin and metalloporphyrin injection on ALAS activity in AIA-treated rats

Porphyrin/Metalloporphyrin	No. of animals	Dose (mg/kg)	ALAS activity (nmole/gm liver/hr.)
No treatment	27		85 ± 9
Heme	2	0.75	80 ± 8
	2	3.0	39 ± 6
Mesoheme	3	1.5	53 ± 7
	5	3.0	48 ± 6
	1	6.0	44 ± 6
Deuteroheme	2	3.0	90 ± 6
Protoporphyrin	2	3.0	88 ± 5
Cobalt Protoporphyrin	2	1.5	50 ± 8
	3	3.0	45 ±10
Heme dimethyl ester (in liposomes)	4	2.5	84 ± 5
FeTPPS	4	2.5	85 ± 5

AIA-treated rats were injected with the metalloporphyrin or porphyrin one hour before sacrifice in volumes of ca. 0.5 ml per rat. All treatments were in PBS alone, with the exception of heme dimethyl ester, which was solubilized by incorporation into PC/SA liposomes. Neither PBS nor PC/SA alone had any effect (not shown).

3 mg/kg heme were administered intravenously. We therefore compared the effectiveness of various heme analogs to that of heme at approximately 3 mg/kg 1 hour after their administration. Deuteroheme (substitution of non-polar groups in positions 2 and 4 with hydrogen atoms) did not inhibit the induction of ALAS activity but mesoheme (substitution of vinyl by ethyl in positions 2 and 4) was almost as effective as heme (Table I). Heme dimethyl ester and FeTPPS caused no inhibition of ALAS activity. Protoporphyrin by itself was also not effective but cobalt protoporphyrin caused an inhibitory effect on ALAS activity paralleling that of mesoheme.

The present study emphasizes that the mechanism leading to heme-mediated inhibition of hepatic ALAS activity is unspecific insofar as Fe-protoporphyrin IX is not the only metalloporphyrin to exert this effect. The mechanism of regulation of ALAS activity is not yet well-delineated. Many investigators envision that a regulatory cytosolic heme pool controls the synthesis and assembly of ALAS in the cytosol and inhibits its uptake by mitochondria (18-21). It has also been proposed that the tissue concentration of metal ions, in particular iron, regulates ALAS activity (22,23). Yet iron appears to be incorporated *in vivo* into heme before being effective, and similarly, $CoCl_2$ injection seems to inhibit ALAS induction only after the formation of Co-protoporphyrin IX, which inhibits as well as does heme (24-26). Results obtained with cultures of chick embryo livers (27) indicate that it is always metalloporphyrins rather than porphyrins or metals alone which are the effective agents, iron- and cobalt-porphyrins being the most active in inhibiting ALAS activity.

The results of the present investigation *in vivo*, examining the short-term effect of selected porphyrins and metalloporphyrins in rats, suggest that for this species it is not only the central iron or cobalt atom but also the porphyrin side chains which determine the structural requirements for the mechanisms operative in inhibiting ALAS activity. Mesoheme, in which ethyl replaces vinyl in positions 2 and 4, proved almost as effective as heme in the present study as well as in a study by Bornheim et al. (28) on the functional

reconstitution of cytochrome P450. Mesoheme is also an excellent substrate for heme oxygenase (29). However, substitution with hydrogen atoms at these two positions (deuteroheme) rendered the porphyrin molecule totally ineffective. It is unlikely that within one hour the ethyl groups could be converted *in vivo* to vinyl groups. Therefore we conclude that, similar to the regulatory effect on the activation of guanylate cyclase activity (30), hydrophobic side chains at positions 2 and 4 and propionic acid residues at positions 6 and 7 (as found in protoporphyrin IX and mesoporphyrin) are essential for the process leading to inhibition of ALAS activity.

It can be speculated that porphyrin-binding proteins of serum and of hepatic cytosol are instrumental in the uptake and intracellular distribution of metalloporphyrins in the liver (31). These proteins bind metalloporphyrins with different affinities and species specificities, which may explain how the metalloporphyrin effects on ALAS activity are not necessarily identical from species to species.

The effectiveness of heme incorporated into liposomes of various compositions was tested and these are shown in Table II. The inhibitory effect on ALAS activity at 1 h post-injection of heme in liposomes was not increased over that achieved by administration of heme in buffer.

Table II: Effect of heme liposome injection on ALAS activity in ALAS treated rats

Liposomes	No. of rats	Heme dose (mg/kg)	ALAS activity (nmole/gm liver/hr)
PC/CP (8:1)	2	0.75	80 ± 7
	7	1.5	48 ± 8
	4	3.0	45 ± 4
PC/CP/chol (8:1:3)	2	1.5	53 ± 6
	2	3.0	40 ± 4
PC/SA (8:1)	1	3.0	40 ± 4
PC/SA/chol (8:1:3)	4	3.0	42 ± 5
PC/SA/chol/LC (12:1:6.5:1.5)	3	2.7	39 ± 4

Treatment was as in Table I; lipid ratios are by weight.

We examined other aspects of heme delivery by liposomes, viz. the possible earlier effectiveness of heme liposomes compared to those given in aqueous medium, and the percentage of hepatic uptake of heme and lipid. As to the former investigation, injections of heme in buffer or in PC/SA/chol liposomes did not inhibit ALAS activity at 15 min after injection but caused a drop in ALAS activity to 50 units at 30 min. Similarly, [^{14}C]heme/[^{3}H]cholesterol in PC/SA/chol liposomes showed a maximal ^{14}C uptake (45% of the injected dose) by the liver at 30 and 60 min after injection. The ^{3}H uptake was 20% at 15 min and remained constant thereafter, possibly because of the rapid exchange with unlabeled cholesterol or excretion of [^{3}H]cholesterol into bile. We also measured the uptake of ^{14}C and ^{3}H in negatively charged liposomes and in those containing the glycolipid lactosylceramide which targets liposomes to the parenchymal liver cells (32). The ^{3}H uptake of lactosylceramide (PC/SA/[^{14}C]heme-[^{3}H]cholesterol/LC) liposomes was 30%, but the ^{14}C uptake was the same as when we injected positively and negatively-charged liposomes which did not contain lactosylceramide. Thus, administration of heme in liposomes causes neither an earlier nor more extensive uptake of heme than if given suspended in aqueous medium. This result, together with the observed rapid removal of heme from liposomes by albumin and hemopexin (14), suggests that heme is removed from liposomes by these two serum proteins before its delivery to the liver *via* the heme-hemopexin complex (33). Liposomes may, however, prevent contact of heme with blood constituents before reaching its place of action and thus prevent its adverse effects on coagulation (10-13).

ACKNOWLEDGMENTS

The work was supported by grants from the American Heart Association-Northeast Ohio Affiliate (to J.B.C.), and the National Institutes of Health (Grant AM-30664 to U. M-E.). The authors would also like to thank Drs. David Marcus, R. F. Pasternack, Arleen Rifkind, Kevin Smith and Martha Kreimer-Birnbaum for helpful discussions and C. Crawford for his contribution in preparing the manuscript.

REFERENCES

1. Granick, S., and Sassa, S. (1971) in *Metabolic Pathways* (H.J. Vogel, ed.), Vol. V, Academic Press, New York, pp. 77-141.
2. Sinclair, P.R., and Granick, S. (1975) Ann. N.Y. Acad. Sci. *244*, 509-520.

3. Kappas, A., Sassa, S., and Anderson, K.E. (1983) in The Metabolic Basis of Inherited Disease, 5th Ed. (J.B. Stanbury et al., eds.), McGraw-Hill, New York, pp. 1301-1384.
4. Yamamoto, M., Hayashi, N., and Kikuchi, G. (1981) Arch. Biochem. Biophys. 209, 451-459.
5. Bonkowsky, H.L., Tschudy, D.P., Collins, A., Doherty, J., Bossenmaier, I., Cardinal, R., and Watson, C.J. (1971) Proc. Natl. Acad. Sci. USA 68, 2725-2729.
6. Watson, C.J., Dhar, G.J., Bossenmaier, I., Cardinal, R., and Petryka, Z.J. (1973) Ann. Intern. Med. 79, 80-83.
7. McColl, K.E.L., Moore, M.R., Thompson, G.G., and Goldberg, A. (1981) Quart. J. Med. New Series L 198, 161-174.
8. Brown, S.B., Shillcock, M., and Jones, P. (1976) Biochem. J. 153, 279-285.
9. Mendenhall, D.W. (1984) N. Engl. J. Med. 311, 539.
10. Morris, D.L., Dudley, M.D., and Pearson, R.D. (1981) Ann. Intern. Med. 95, 700-701.
11. Glueck, R., Green, D., Cohen, I., and Ts'ao, C-H. (1983) Blood 61, 243-249.
12. Neely, S.M., Gardner, D.V., Reynolds, N., Green, D., and Ts'ao, C-H. (1984) Br. J. Haematol. 58, 305-316.
13. Becker, C.G., Wagner, M., Kaplan, A.P., Silverberg, M., Grady, R.W., Liem, H., and Muller-Eberhard, U. (1985) J. Clin. Invest., in press.
14. Cannon, J.B., Kuo, F-S., Pasternack, R.F., Wong, N.M., and Muller-Eberhard, U. (1984) Biochemistry 23, 3715-3721.
15. Fuhrhop, J-H., and Smith, K.M. (1975) in Porphyrins and Metalloporphyrins (K.M. Smith, ed.), Elsevier/North Holland, Amsterdam: a) p. 803; b) p. 773; c) p. 804.
16. Vogel, W., Richert, D.A., Pixley, B.Q., and Schulman, M.P. (1960) J. Biol. Chem. 235, 1769-1775.
17. Sassa, S., Kappas, A., Bernstein, S.E., and Alvares, A.P. (1979) J. Biol. Chem. 254, 729-735.
18. Ades, I.Z. (1983) Biochem. Biophys. Res. Commun. 110, 42-47.
19. De Matteis, F. (1978) in Heme and Hemoproteins (F. De Matteis and W.N. Aldridge, eds.), Springer-Verlag, New York, pp. 129-155.
20. Badawy, A.A-B. (1978) Biochem. J. 172, 487-494.
21. Yamauchi, K., Hayashi, N., and Kikuchi, G. (1980) J. Biol. Chem. 255, 1746-1751.
22. Granick, S. (1966) J. Biol. Chem. 241, 1359-1375.
23. Maines, M.D., and Kappas, A. (1977) Science 198, 1215-1221.
24. Sinclair, P., Gibbs, A.H., Sinclair, J.F., and De Matteis, F. (1979) Biochem. J. 178, 529-538.
25. Igarashi, J., Hayashi, N., and Kikuchi, G. (1978) J. Biochem. (Tokyo) 84, 997-1000.
26. Watkins, S., Baron, J., and Tephly, T.R. (1980) Biochem. Pharmacol. 29, 2319-2323.
27. Schoenfeld, N., Wysenbeek, A.J., Greenblat, Y., Epstein, O., Atsmon, A., and Tschudy, D.P. (1984) Biochem. Pharmacol. 33, 2783-2788.
28. Bornheim, L.M., Correia, M.A., and Smith, K.M. (1984) Biochem. Biophys. Res. Commun. 121, 95-101.
29. Tenhunen, R., Marver, H.S., and Schmid, R. (1969) J. Biol. Chem. 244, 6388-6394.
30. Ignarro, L.J., Ballot, B., and Wood, K.S. (1984) J. Biol. Chem. 259, 6201-6207.
31. Muller-Eberhard, U., and Vincent, S.H. (1985) Biochem. Pharmacol. 34, 719-725.
32. Spanjer, H.H., and Scherphof, G.L. (1983) Biochim. Biophys. Acta 734, 40-47.
33. Smith, A., and Morgan, W.T. (1981) J. Biol. Chem. 256, 10902-10909.