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THE EFFECTS OF METALLOPORPHYRINS, PORPHYRINS AND METALS ON THE ACTIVITY OF DELTA-AMINOLEVULINIC ACID SYNTHASE IN MONOLAYERS OF CHICK EMBRYO LIVER CELLS

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Abstract—The effect of various metals, porphyrins and metalloprphyrins on the activity of delta-aminolevulinate synthase (ALAS) was measured in monolayers of chick embryo liver cells in order to determine whether the metal moiety of heme or heme itself is the regulator of ALAS activity.

Iron, magnesium, zinc, copper, manganese and nickel did not decrease ALAS activity in non-induced and in cells induced by allyl-isopropylacetamide (AIA). Cobalt decreased both non-induced and induced activity. Porphyrins inhibited ALAS, apparently only after having been converted into metalloporphyrins. Almost all the metalloporphyrins examined decreased ALAS activity. None of the substances, at the concentrations used, was toxic to the cells.

These observations indicate that probably heme and not iron is the regulator of ALAS activity in monolayers of chick embryo liver cells.

The first and rate-limiting step in heme biosynthesis is catalyzed by delta-aminolevulinic acid synthase (ALAS), a mitochondrial enzyme with a very rapid turnover rate [1, 2].

Most investigators assume the existence of an intracellular free heme pool, the concentration of which regulates ALAS activity [3–6]. Others believe that the concentration of free iron regulates ALAS activity [7–11]. Maines and Kappas suggest that the iron atom of heme is the active regulator of ALAS and of heme oxygenase, and that the tetrapyrrole moiety of the heme complex functions as a means of transport of the metal to regulatory sites in the cells [8]. According to this concept only the ionic form of metals such as Co^{2+} appears capable of altering heme metabolism [8]. On the other hand, Sinclair *et al.* provided evidence for the formation of Co-protoporphyrin in the livers of rats treated with $CoCl_2$ *in vivo* [12] and in chick embryo liver cells *in vitro* [13, 14], and suggested the direct involvement of Co-protoporphyrin in the inhibition of ALAS observed in the livers of treated animals. Igarashi *et al.* [15] showed that in the rat Co-protoporphyrin is formed from Co^{2+}, and that Co-protoporphyrin inhibits drug-induced increases of ALAS, as well as its transfer from the cytosol into the mitochondrion. Watkins *et al.* [16] also showed the production of Co-protoporphyrin from $CoCl_2$ in rat liver.

This study was undertaken in order to investigate the role of various metalloporphyrins and their metal and tetrapyrrole moieties on the regulation of non-induced and induced activity of ALAS in chick embryo liver cells.

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MATERIALS AND METHODS

Metalloporphyrins and porphyrins were obtained from Porphyrin Products, Logan, UT and from Sigma Chemicals, St. Louis, MO. Delta-aminolevulinic acid (ALA) was obtained from Sigma Chemicals. Collagenase and hyaluronidase were purchased from Grand Island Biological Co., Grand Island, NY. Allyl-isopropylacetamide (AIA) was a gift from Hoffman La-Roche, Nutley, NJ. Modified Ham's F-12 medium [17] was purchased from the Central Biology Laboratory, Haim Sheba Medical Center, Tel Hashomer and The Biological Industries, Bet Haemek, Israel. All other chemicals were of reagent grade.

Cell cultures of chick embryo liver cells were prepared according to Sassa and Kappas [17], with minor modifications. The cells were suspended in 100 vol of modified Ham's F-12 medium, without serum, with insulin, hydrocortisone and triiodothyronine [17]. Two milliliter or 9 ml portions of this suspension were added to culture dishes of 3.5 or 9 cm diameter, respectively (Nunclon, Rosekilde, Denmark). After 24 hr of incubation at 37° in an atmosphere of 5% CO_2 and 95% air the medium was replaced by fresh medium as above. At this time the various chemicals were added to the culture media with or without AIA, 60 μg/ml medium (0.43 mM), and incubation was continued for an additional 18 hr. When ALAS activity was to be determined by the indirect method

(vide infra) levulinate, 25 mM, and glycine, 5 mM, were also added at the time of replacement of the medium. For direct determination of ALAS (vide infra) culture dishes of 9 cm diameter with 9 ml cell suspensions were used.

The method of Burnham (personal communication) was used for dissolving the various metalloporphyrins, porphyrins and metals in a mixture containing 7.25% 1 N NH_4OH, 35.5% H_2O, 50% absolute ETOH, 7.25% Tris 1 M, pH 8.0. Preliminary experiments showed that the addition of 20 μl of the alkaline solvent to cultures of cells did not change the pH of the medium and had no influence on the formation of monolayers or the activity of non-induced or induced ALAS.

For determination of ALAS activity two different methods were employed. The first was the indirect method of Sinclair and Granick (Method I) [18], in which the amount of ALA accumulated in medium and cells is measured after 18–24 hr of incubation in the presence of 25 mM levulinate and 5 mM glycine. Control values obtained with this method in non-induced and in AIA-induced cells were 8–10 and 26–28 nmole ALA/mg protein/18 hr, respectively. It was observed that addition of 25 mM levulinate to the monolayers caused a slight to two-fold increase in ALAS activity, as described by Sinclair and Granick [19].

The second method used was a direct measurement of ALAS activity in homogenates of cells, as described by Sinclair and Granick [18]. Control values obtained with this method in non-induced and in AIA-induced cells were 1–1.5 and 4–6 nmole ALA/mg protein/60 min, respectively.

Two methods were employed to determine the content of heme in the cells, the fluorometric "hot oxalic method" [20] and the "pyridine hemochromagen method" [21]. The concentration of Zn-protoporphyrin was determined according to Chisolm *et al.* [22].

Determination of the activity of tyrosine aminotransferase (TAT), both non-induced and induced by dexamethasone, was performed according to Diamondstone [23] and Whitlock [24], as modified by us [25]. The incorporation of ^{14}C-leucine, ^{14}C-uridine and ^{14}C-thymidine into protein, RNA and DNA respectively was determined as described previously [26, 27]. Statistical evaluations were performed according to the following tests: Student's *t*-test, *t*-paired comparison test, one way analysis of variance followed by an aposteriori test, Sum of Squares Stimulation Test Procedure (SS-STP) and two way analysis of variance with replications [28].

RESULTS

At the outset of these investigations it was decided to determine changes in ALAS activity by measuring the accumulated ALA in the medium and in the cells of monolayers of chick embryo liver cells during 18 hr of incubation according to the indirect method of Sinclair and Granick [18]. The incubation takes place in the presence of 25 mM levulinate, which partially inhibits delta-aminolevulinate dehydrase (ALAD), thus causing accumulation of ALA. The advantage of this method is its simplicity as compared to direct measurements of ALAS activity in cellular homogenates. In order to reascertain its validity a comparison of the two methods was performed with some of the substances investigated. The results are shown in Table 1 and indicate that there is no significant difference between the percentage of inhibition obtained by the two methods according to the *t*-paired comparison test.

Table 1. Changes in ALAS activity measured by two different methods

Addition, 10^{-5} M final concentration	Indirect method (% change)	Direct method (% change)
$MnCl_2$	+109	+109
Mn-protoporphyrin	−25	−23
Protoporphyrin	−61	−59
$CoSO_4$	−45	−33
Co-protoporphyrin	−96	−91
$FeSO_4$	0	−3

Both methods were performed according to Sinclair and Granick [18]. Eighteen hours before the measurements various substances and AIA, 60 μg/ml, were added to the monolayers. Monolayers with AIA and the solvent used for dissolving the various additions served as controls and the values obtained were regarded as 100%. The values were: 27 ± 1 nmole ALA/mg protein/18 hr in the indirect method and 5 ± 1 nmole ALA/mg protein/60 min in the direct method. The *t*-paired comparison test was employed to examine the difference between the two methods used.

In accordance with these results all the measurements of ALAS activity were carried out with the indirect method.

The non-induced and the induced activities of ALAS were determined in the presence of metals, porphyrins and metalloporphyrins. The results are shown in Table 2.

Protoporphyrin, mesoporphyrin and deuteroporphyrin at a concentration of 10^{-5} M reduced both induced and non-induced ALAS activity by about 50–60%.

The metals: iron, magnesium, zinc, copper, manganese and nickel did not have any effect on the non-induced activity of ALAS. Iron, magnesium and zinc had also no effect on the induced ALAS activity. Copper, manganese and nickel augmented ALAS-induced activity. Cobalt was found to be the only metal which reduced both non-induced and induced activity of ALAS.

Almost all the metalloporphyrins tested reduced both non-induced and induced ALAS activity at a concentration of 10^{-5} M (Table 2). The strongest inhibitors decreased ALAS activity also at 10^{-6} and 10^{-7} M (not shown).

The effects of porphyrins and of metalloporphyrins at a concentration of 10^{-4} M were generally similar to those at 10^{-5} M, but minor cell toxicity was observed by the Trypan Blue exclusion test. This test was negative when cells were treated with these substances at 10^{-5} M.

In order to determine whether porphyrins and metalloporphyrins inhibit directly ALAS activity protoporphyrins, Fe-protoporphyrin, Mn-protoporphyrin and Co-protoporphyrin, each at 10^{-5} M final concentration, were added to cell homogenates. No

Table 2. The effects of metalloporphyrins, porphyrins and metals on the induced and non-induced activity of ALAS in monolayers of chick embryo liver cells

Porphyrins and metalloporphyrins	ALAS activity, (nmole ALA/mg protein/18 hr) non-induced	induced
—	8.1 ± 0.9	24.0 ± 1.5
Protoporphyrin	4.4 ± 0.2*	9.4 ± 1.4*
Fe-protoporphyrin	4.5 ± 0.1*	9.4 ± 0.2*
Mg-protoporphyrin	4.6 ± 0.2*	10.7 ± 1.5*
Zn-protoporphyrin	3.6 ± 0.8*	9.4 ± 1.5*
Cu-protoporphyrin	6.4 ± 0.2*	21.1 ± 0.1*
Mn-protoporphyrin	9.1 ± 0.5	21.1 ± 2.1
Co-protoporphyrin	3.8 ± 0.9*	0.5 ± 0.2*
Ni-protoporphyrin	4.6 ± 1.0*	18.0 ± 1.5*
Mesoporphyrin	3.8 ± 0.2*	7.4 ± 1.2*
Fe-mesoporphyrin	3.7 ± 0.8*	10.7 ± 4.3*
Mg-mesoporphyrin	4.9 ± 0.9*	7.9 ± 3.1*
Zn-mesoporphyrin	4.0 ± 0.2*	11.0 ± 0.4*
Cu-mesoporphyrin	5.9 ± 0.6*	20.7 ± 0.3*
Mn-mesoporphyrin	9.6 ± 0.3*	22.2 ± 2.4
Co-mesoporphyrin	3.2 ± 0.1*	1.9 ± 2.2*
Ni-mesoporphyrin	2.9 ± 0.5*	16.0 ± 0.4*
Deuteroporphyrin	3.8 ± 0.3*	10.1 ± 1.2*
Fe-deuteroporphyrin	5.8 ± 0.5*	11.2 ± 0.4*
Zn-deuteroporphyrin	4.3 ± 0.6*	14.1 ± 0.2*
Cu-deuteroporphyrin	6.4 ± 0.1*	18.5 ± 0.2*
Mn-deuteroporphyrin	9.5 ± 0.4*	18.5 ± 2.3*
Co-deuteroporphyrin	2.7 ± 1.7*	0.7 ± 0.6*
Ni-deuteroporphyrin	6.6 ± 0.1*	16.6 ± 0.9*
Hematoporphyrin	6.4 ± 0.3*	9.8 ± 2.1*
Metals		
$FeSO_4$ or $FeCl_3$	8.0 ± 0.7	25.2 ± 2.1
$MgSO_4$	8.4 ± 0.9	24.1 ± 1.5
$ZnSO_4$	8.2 ± 0.6	23.0 ± 2.1
$CuSO_4$	8.0 ± 0.7	38.0 ± 1.2*
$MnCl_2$ or $MnSO_4$	7.9 ± 0.8	54.4 ± 4.8*
$CoSO_4$ or $CoCl_2$	6.0 ± 0.3*	14.4 ± 1.2*
$NiCl_2$	7.6 ± 0.1	27.8 ± 1.3*

The final concentration of each of the various additions was 10^{-5} M. Induction was carried out with AIA, 60 μg/ml. ALAS activity was measured by the indirect method. The means and standard deviations are based upon 6 determinations in 3 separate experiments. Asterisks indicate a significant difference ($P < 0.05$) as determined by Student's *t*-test.

changes in ALAS activity were observed (not shown).

Evaluation of the data shown in Table 2 according to a two way analysis of variance with replications showed that the inhibitory effect of metalloporphyrins on ALAS activity is not the result of the additive effects of their constituents—the metal and porphyrin ($P < 0.05$).

Since in these experiments the metals and the porphyrins were examined separately, the effects of the simultaneous addition of both a metal salt and a porphyrin, each at 10^{-5} M final concentration, were compared to the effect of the relevant metalloporphyrin at 10^{-5} M. The results are shown in Table 3.

A one way analysis of variance followed by an aposteriori test (SS-STP) was employed to determine whether there is a significant difference between the effects of the mixtures of pophyrins and metals and the relevant complexes. The results indicate that the inhibition of ALAS activity caused by a metalloporphyrin is significantly different from that obtained by a mixture of its constituents. The only exception found was the combination of cobalt and porphyrin compared to Co-porphyrin. The latter observation is discussed later.

In order to determine whether the influence of porphyrins on ALAS activity is caused by the porphyrins themselves or by their conversion into metalloporphyrins, ALAS activity was measured after addition of both porphyrin and desferrioxamine to the culture medium. Desferrioxamine binds iron and, therefore, prevents the formation of iron-porphyrins. Under these conditions the various porphyrins: proto-, meso- and deuteroporphyrin did not inhibit ALAS induction. The presence of des-

Table 3. Effects of the combination of metals and porphyrins and of metalloporphyrins on induced ALAS activity in monolayers of chick embryo liver cells

	(%) Change in ALAS activity	
	Metal and porphyrin	Metalloporphyrin
Zn and protoporphyrin	−42.0 ± 4.0	−61.0 ± 1.0*
Cu and protoporphyrin	−36.0 ± 0.5	−11.5 ± 0.5*
Mn and protoporphyrin	−28.0 ± 3.0	−12.0 ± 9.2*
Co and protoporphyrin	−97.0 ± 1.0	−98.0 ± 1.0
Ni and protoporphyrin	−45.0 ± 1.0	−25.0 ± 6.3*
Cu and mesoporphyrin	−44.0 ± 8.0	−13.5 ± 1.5*
Mn and mesoporphyrin	−27.5 ± 5.5	−7.5 ± 11.3*
Co and deuteroporphyrin	−94.0 ± 1.0	−97.3 ± 2.5

Metals and porphyrins or metalloporphyrins were added to the culture media, each at a final concentration of 10^{-5} M. Induction was carried out with AIA, 60 μg/ml. After 18 hr incubation ALAS activity was determined by the indirect method. ALAS activity in the control cultures was 24.6 ± 1.4 nmole/mg protein/18 hr, and considered as 100%. Asterisks indicate a significant difference according to a single classification analysis of variance followed by an aposteriori test (SS-STP). The test was performed on the numerical values of ALAS activity. Each pair of values obtained in the presence of either the mixture of metal and porphyrin or the complex was compared to the control value.

ferrioxamine did not affect the inhibition obtained by Fe- and Co-protoporphyrin (Table 4).

The absence of an inhibitory effect of porphyrins on the induction of ALAS was also observed when another inhibitor of ferrochelatase activity, ascorbic acid, 10 mM [29], was added to the medium instead of desferrioxamine (not shown). The results would seem to indicate that porphyrins reduce ALAS activity only after their conversion to metalloporphyrins.

The time related changes in ALAS activity of cultures exposed to protoporphyrin (Fig. 1) are in accordance with the above hypothesis.

The figure shows that addition of protoporphyrin to AIA leads to a rapid rise in cellular heme, above that caused by AIA only, from about 300 to 700 pmole/mg protein. The decrease in ALAS activity in the presence of AIA and protoporphyrin compared to that of cultures treated with AIA only, occurred after cellular heme had started to increase. The time relation between the changes in these parameters are in accordance with the half-life of ALAS and indicate that an inhibitory effect on the induction of ALAS takes place only after the transformation of protoporphyrin to heme.

Because of the high intracellular concentrations

Table 4. The effect of porphyrins on the induced activity of ALAS in the presence of desferrioxamine

Treatment	ALAS Activity (%)
—	100
protoporphyrin	39
deuteroporphyrin	42
mesoporphyrin	40
Fe-protoporphyrin	38
Co-protoporphyrin	3
desferrioxamine	279
protoporphyrin and desferrioxamine	275
deuteroporphyrin and desferrioxamine	270
mesoporphyrin and desferrioxamine	265
Fe-protoporphyrin and desferrioxamine	36
Co-protophoryin and desferrioxamine	2

Monolayers of chick embryo liver cells were incubated in the presence of AIA, 60 μg/ml. ALAS activity in the presence of AIA only, measured by the indirect method, was 24.1 nmole ALA/mg protein/18 hr and was considered as 100%. The various porphyrins and metalloporphyrins were added to the medium in a final concentration of 10^{-5} M. Desferrioxamine was present in a concentration of 1.5 mM. The data are the means of results obtained in two separate experiments.

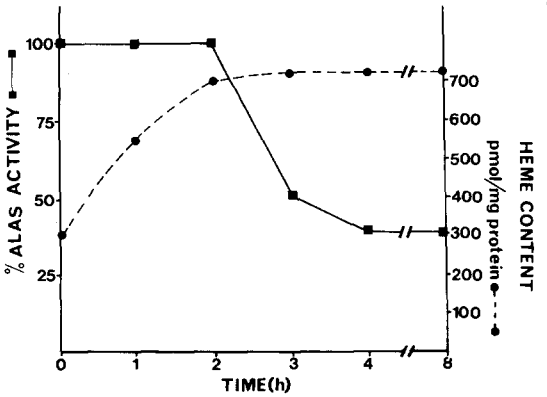

Fig. 1. The effect of duration of incubation with protoporphyrin on ALAS activity and heme content. Monolayers were incubated in the presence of AIA, 60 μg/ml, or AIA, 60 μg/ml and protoporphyrin, 10^{-5} M, added at time 0. ALAS activity in cultures treated with AIA only was considered as 100% at each of the time points indicated. The percent change in ALAS activity in cultures treated with AIA and protoporphyrin was calculated accordingly, at each of the time points indicated. ALAS activity at 0 time was 0.5 nmole ALA/mg protein/30 min. The content of intracellular heme was determined at each of the time points indicated in cultures treated with AIA, 60 μg/ml, and protoporphyrin, 10^{-5} M. The data are the means of results obtained in two separate experiments.

of protoporphyrin in these experiments heme was determined both by the fluorometric and the pyridine hemochromogen methods. The results obtained were similar.

The influence of protoporphyrin, Fe-protoporphyrin, Mn-protoporphyrin and Co-protoporphyrin at 10^{-5} M, on ^{14}C-leucine, ^{14}C-uridine and ^{14}C-thymidine incorporation into protein, RNA and DNA, respectively, was examined. No effects were observed (not shown), indicating that the inhibition of ALAS by these substances under the conditions examined is not related to these parameters.

In another series of experiments it was found that the various porphyrins mentioned above do not affect the dexamethasone induction of TAT, an enzyme which is not related to the porphyrin biosynthetic pathway (not shown).

DISCUSSION

The details of the physiological regulation of ALAS activity have not been solved satisfactorily. Most investigators assume that the concentration of heme in a "free" or "loosely bound" heme pool is the major determinant factor in the control of ALAS synthesis through the operation of a closed negative feedback loop [for reviews see 5, 6, 19]. In contrast, a central role of metal ions in this metabolic control has been proposed [reviewed in 8]. The arguments in favor of this hypothesis have never been completely and satisfactorily refuted.

Most of the experiments which indicate the central role of metal ions in the regulation of ALAS activity were carried out by injecting metal salts into rats. We thought that a thorough simultaneous examination of the influence of metals, porphyrins and metalloporphyrins in the simple system of monolayers of chick embryo liver cells might contribute to the solution of the controversy.

According to the data shown in this investigation the seven metals examined at 10^{-5} M, zinc, iron, nickel, magnesium, manganese, copper and cobalt, cannot be regarded as regulators of ALAS activity. Four of these, zinc, iron, nickel and magnesium, did not have any effect; manganese and copper increased AIA induced ALAS activity but had no effect on the non-induced enzyme. Only cobalt was found to decrease both induced and non-induced ALAS activity. However, cobalt is converted to cobalt-protoporphyrin [12–16] and apparently inhibits ALAS activity only in the latter form, as shown by Sinclair *et al.* [13, 14] and by us [30].

The porphyrins tested decreased both non-induced and induced activity of ALAS to the same extent, about 50% at 10^{-5} M.

This investigation showed that porphyrins themselves have no direct effect on ALAS activity as was reported already by Granick [1] and that they are apparently effective only after having been converted to metalloporphyrins.

Almost all the metalloporphyrins tested decreased both non-induced and induced activity of ALAS and the degree of inhibition obtained by the various metalloporphyrins is determined by the metals and not by the porphyrin moieties examined. A decrease in ALAS activity by metalloporphyrins was reported previously both *in vivo* [31–33] and *in vitro* [1].

Evaluation of the results according to a two way analysis of variance with replications shows that the influence of a metalloporphyrin on ALAS activity is not the result of the additive effects of its constituents, but a result of an interaction between the metal and the porphyrin.

All the substances which clearly inhibited both non-induced and induced ALAS activity, cobalt, porphyrins and various metalloporphyrins at 10^{-5} M, did not influence induction of tyrosine-aminotransferase by dexamethasone and incorporation of ^{14}C-leucine, ^{14}C-uridine and ^{14}C-thymidine into protein, RNA and DNA, respectively. Moreover, Trypan-Blue exclusion tests were negative. It may, therefore, be concluded that at 10^{-5} M none of the substances examined had a toxic influence on the cells and that the inhibition of ALAS activity is a specific effect.

On the basis of the observations described we conclude that metalloporphyrins rather than free metals probably regulate ALAS activity in chick embryo liver cells in culture. Extrapolating from these conclusions we suggest that also in vivo heme and not iron regulates ALAS activity.

Note added in proof. After submitting our article for publication the following relevant article, which states that heme and not iron is the regulator of ALAS activity *in vivo*, was brought to our attention: K. T. Kitchin, Regulation of rat hepatic delta-aminolevulinic acid synthatase and heme oxygenase activities: evidence for control by heme and against mediation by prosthetic iron. *Int. J. Biochem.* **15** 479 (1983).

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