The effect of *N*-methylprotoporphyrin and succinyl-acetone on the regulation of heme biosynthesis in chicken hepatocytes in culture

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The essential features of hepatic protoporphyria, namely inhibition of ferrochelatase, accumulation of protoporphyrin and stimulation of 5-aminolevulinic acid synthase (ALA-S) were all obtained by treating chicken hepatocytes in culture with small doses of *N*-methylprotoporphyrin. Both *N*-methylprotoporphyrin and succinyl-acetone, another inhibitor of heme biosynthesis, stimulated ALA-S when given on their own and also enhanced the stimulation of ALA-S caused by phenobarbital.

Porphyria *Heme* N-*Methylprotoporphyrin* *5-Aminolevulinic acid synthase* *3,5-Diethoxycarbonyl-1,4-dihydrocollidine* *(Liver)*

1. INTRODUCTION

3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) has long been known to cause a marked inhibition of hepatic ferrochelatase (EC 4.99.1.1), leading to accumulation of protoporphyrin and to the biochemical picture of hepatic protoporphyria [1]. Stimulation of the first and rate-limiting enzyme of the pathway, 5-aminolevulinic acid synthase, ALA-S (EC 2.3.1.37) is also observed after DDC treatment [2], an effect which contributes significantly to the accumulation of protoporphyrin [3]. DDC has also been reported to potentiate markedly the stimulation of ALA-S activity caused in the liver by a large number of lipid-soluble drugs, including phenobarbital [4–6]. It has been proposed that these effects of DDC on ALA-S activity may be secondary to the inhibition of ferrochelatase, since the decreased heme formation thus resulting would lead to diminished heme-mediated feedback repression of ALA-S and, therefore, to increased ALA-S activity [3–5,7].

In [8,9], a powerful inhibitor of ferrochelatase has been extracted from the liver of animals treated with DDC. The inhibitor has been identified as *N*-methylprotoporphyrin [10–12] and has been shown to originate from metabolic transfer of the intact 4-methyl group of DDC to one of the pyrrole nitrogens of the porphyrin moiety of liver heme [12–14]. Confirmation of the inhibitory effect on ferrochelatase has been obtained using synthetic *N*-methylated porphyrins [10,15–17].

N-Methylprotoporphyrin was shown to share the ability of DDC to markedly inhibit ferrochelatase activity in chick embryo liver cell culture [17]. However, unlike DDC, *N*-methylprotoporphyrin did not elevate ALA-S when measured 12 h after addition to the hepatocyte culture. It was therefore concluded that *N*-methylprotoporphyrin-induced ferrochelatase inhibition was not accompanied by enhanced ALA-S activity.

We have now investigated the effect of authentic *N*-methylprotoporphyrin on ALA-S activity at additional time periods in cultured chicken

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hepatocytes. We found that inhibition of ferrochelatase, accumulation of protoporphyrin and compensatory stimulation of ALA-S are all obtained 5 h after addition of the alkylated porphyrin, in keeping with the concept that conversion of liver heme to the inhibitory *N*-methylprotoporphyrin is the primary biochemical mechanism by which DDC induces hepatic porphyria. The effect of succinyl-acetone, another inhibitor of heme biosynthesis [18,19], has also been investigated.

2. MATERIALS AND METHODS

2.1. *Chick embryo liver cell cultures*

The details of the cell culture technique have been described [20,21]. The hepatocytes were maintained in serum-free Waymouth MD705/1 medium supplemented with penicillin G, streptomycin, insulin and thyroxine, in 10 cm diam. disposable plastic dishes containing 15 ml medium. After an initial incubation of 24 h, the medium was discarded, replaced with fresh medium and the various drugs added. Phenobarbital sodium and succinyl-acetone were added dissolved in saline (20 μl/dish) and *N*-methylprotoporphyrin (free acid), dissolved in 95% ethanol (20 μl/dish), control dishes receiving the appropriate solvent only. After a further incubation of either 5 h or 24 h, the medium was discarded and 5 ml of a solution containing 0.9% NaCl, 0.01 M Tris–HCl and 0.5 mM EDTA at pH 7.4 were added to each dish. The cells were removed by scraping with a rubber policeman and cells from two identically treated dishes were combined and centrifuged (500 $\times$ *g*) for 5 min. The cell pellet was suspended in 0.5–2 ml ice-cold NaCl–Tris–EDTA buffer and homogenized using a Potter-type homogenizer. Homogenates for ferrochelatase assay were prepared in ice-cold Tris–HCl buffer (pH 8.2) using a Polytron homogenizer.

2.2. *Measurement of enzyme activities*

Ferrochelatase activity of the cell homogenates was measured by the pyridine hemochromogen method, using mesoporphyrin and iron as substrates, as in [17]. ALA-S activity was measured by a slight modification of an isotopic technique [22]: cell homogenates (equivalent to 0.5–1.5 mg protein) were incubated for 30 min at 38°C in air but without shaking, in the presence of Tris–HCl buffer (63 mM), glycine (80 mM), EDTA (8 mM), pyridoxal-5′-phosphate (0.4 mM) and [2,3-^{14}C]succinate (0.37 mM), in a total volume of 0.5 ml, at pH 7.2. 5-Aminolevulinic acid dehydratase (EC 4.2.1.24) was assayed as in [23], using the Ehrlich reagent, 2 M with respect to $HClO_4$ [24], to estimate the porphobilinogen formed.

2.3. *Estimation of porphyrins and of protein*

Total cell porphyrins were extracted into 20 vol. 0.9 N $HClO_4$–ethanol (1:1) and measured fluorimetrically using a Turner instrument calibrated with appropriate standards of coproporphyrin III [25]. Protein was assayed by a modification [26] of the Lowry [27] procedure.

2.4. *Source of special chemicals*

N-Methylprotoporphyrin dimethyl ester was prepared from protoporphyrin IX dimethylester as in [13] except that the reaction was carried out at 100°C for 4 h. The concentration of the methylester was first determined using an E_{mM} = 127.8 [28] for its Soret absorption in $CHCl_3$; the ester was then hydrolysed with 6 M HCl for 16 h and the resulting free acid was dried under N_2, dissolved in 95% ethanol and stored in the dark at −70°C. Succinyl-acetone (4,6-dioxoheptanoic acid) was obtained from Calbiochem-Behring (La Jolla, CA 92037).

3. RESULTS AND DISCUSSION

When chicken hepatocytes in culture were treated for 5 h with increasing amounts of *N*-methylprotoporphyrin, a very pronounced inhibition of ferrochelatase was found and even the lowest dose of alkylated porphyrin (6 ng/ml of medium) produced maximal or nearly maximal loss of enzyme activity (fig.1). A marked increase in cellular porphyrins (mostly protoporphyrin as determined by fluorescence spectroscopy) was seen and, in addition, a significant stimulation of ALA-S. At higher concentrations of *N*-methylprotoporphyrin further increases in ALA-S activity were found and these were accompanied by corresponding further increases in cell porphyrin concentration (fig.1). A stimulation of ALA-S activity was also seen 5 h after treating hepatocytes with

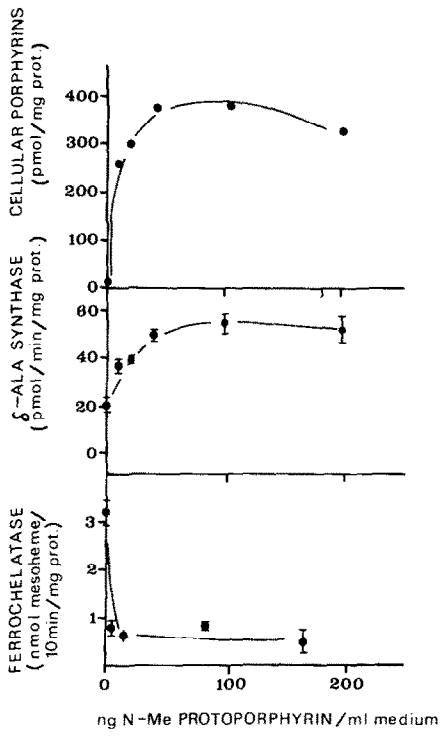

Fig.1. Effect of increasing doses of *N*-methylprotoporphyrin on ALA-S and ferrochelatase activities and on total porphyrin content of cultured hepatocytes, 5 h after treatment. Average ± SEM of at least 3 observations or average of 2 observations: * $p \leqslant 0.01$; ** $p \leqslant 0.001$, when compared with corresponding control value.

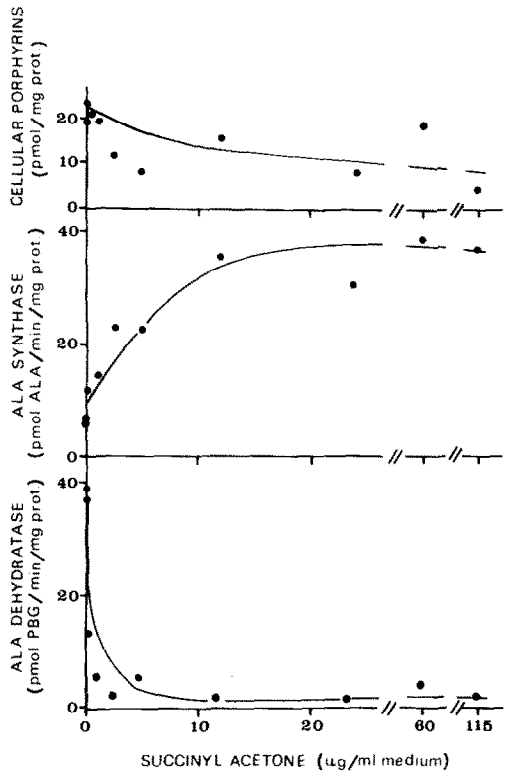

Fig.2. Effect of increasing doses of succinyl-acetone on the activities of ALA-S and 5-aminolevulinic acid dehydratase and on total porphyrin content of cultured hepatocytes, 5 h after treatment. Single observations each obtained with pooled hepatocytes from two identically treated dishes.

succinyl-acetone (fig.2), another inhibitor of heme biosynthesis. In this case the expected loss of activity of 5-aminolevulinic acid dehydratase, the specific target of succinyl-acetone inhibition [19], could also be demonstrated (fig.2), but no rise in cellular porphyrins was found.

N-Methylprotoporphyrin and succinyl-acetone not only stimulated ALA-S when given on their own but also enhanced the stimulation of ALA-S activity caused by a lipid-soluble inducer, phenobarbital (table 1). Succinyl-acetone was more effective than *N*-methylprotoporphyrin in both respects; in addition, a stimulation of ALA-S was still observed 24 h after addition of succinyl-acetone, whereas a depression of ALA-S activity (below the level of the corresponding control value) was seen 24 h after *N*-methylprotoporphyrin (table 1). This suggests that *N*-methylprotoporphyrin may possess two opposite effects on ALA-S, one leading to stimulation, the other to depression of synthase activity, the first effect predominating at the early time point. The mechanism by which *N*-methylprotoporphyrin causes a late inhibition of ALA-S is not known.

The finding that two inhibitors of heme biosynthesis can enhance the stimulation of ALA-S caused by phenobarbital is compatible with the concept [4–7,29–32] that when the negative feedback control exercised by heme on ALA-S is diminished, the enzyme becomes more sensitive to stimulation by lipid-soluble drugs. This work also indicates that inhibition of heme biosynthesis can alone lead to a compensatory stimulation of ALA-S (fig.1,2; table 1). While an inducer with high lipid-solubility

Table 1

Effect of *N*-methyl protoporphyrin (*N*-MePP), succinyl-acetone (SA) and sodium phenobarbital (PB) on the activity of ALA-S and total porphyrin content of chicken hepatocytes in culture

Drug added and initial concentration (μg/ml medium)	ALA-S (pmol ALA.min^{-1}.mg prot.$^{-1}$)	Total porphyrins (pmol/mg protein)
5 h Treatment		
Control	12.9 ± 1.1 (18)	19.0 ± 2.6 (21)
N-MePP (0.2)	30.6 ± 2.8 (11)[b]	438.0 ± 33.5 (12)[b]
SA (115.0)	45.6 ± 2.0 (6)[b]	16.6 ± 3.9 (7)
PB (50.0)	19.5 ± 1.1 (10)[b]	15.5 ± 2.7 (12)
N-MePP (0.2) + PB (50.0)	51.7 ± 7.4 (6)[c]	532.1 ± 77 (8)
SA (115.0) + PB (50.0)	66.0 ± 5 (3)[c]	14.2 ± 4.6 (4)
24 h Treatment		
Control	8.2 ± 0.87 (9)	18.7 ± 3.8 (11)
N-MePP (0.2)	5.3 ± 0.7 (7)[a]	433.0 ± 33 (8)[b]
SA (115.0)	19.2 ± 3.5 (3)[a]	21.9 ± 5.4 (3)

[a] $p \leqslant 0.05$
[b] $p \leqslant 0.001$, when compared with corresponding control value
[c] $p \leqslant 0.02$, when compared with corresponding PB value

Results given are averages ± SEM of the number of observations in parentheses

(e.g., phenobarbital) will enhance ALA-S induction, its presence is not obligatory (table 1). Thus a state of heme depletion is a sufficient stimulus for induction of ALA-S [33].

The essential features of hepatic protoporphyria, namely inhibition of ferrochelatase, accumulation of protoporphyrin and stimulation of ALA-S were all obtained after addition of *N*-methylprotoporphyrin to chicken hepatocytes in culture; this provides strong support for the hypothesis that production of *N*-methylprotoporphyrin from liver heme is the primary mechanism by which DDC induces hepatic porphyria.

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