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δ-Aminolaevulinate synthase in human HepG2 hepatoma cells

Repression by haemin and induction by chemicals

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δ-Aminolaevulinate (ALA) synthase, the rate-limiting enzyme in haem biosynthesis in the normal liver, was examined in human HepG2 hepatoma cells. Haemin, up to 100 μM, had no effect on ALA synthase activity *in vitro*; it did, however, exhibit a dose-dependent inhibitory action when added to cells growing in culture (half-maximal inhibition at 1 μM). The half-life of ALA synthase activity after haemin treatment was 2 h, which was similar to that found after treatment with cycloheximide. Cells treated with actinomycin D showed a longer half-life of the enzyme activity, i.e. 4 h, compared with haemin or cycloheximide treatment. Treatment of cells with succinylacetone markedly inhibited the activity of ALA dehydratase and ^{59}Fe incorporation into haem, but it increased ALA synthase activity. Both the haemin-induced repression and the succinylacetone-mediated de-repression of ALA synthase activity were reversible within 4 h after replacing the medium with fresh medium without the chemical. In addition to succinylacetone, dimethyl sulphoxide and 3-methylcholanthrene induced the enzyme. Induction of ALA synthase by these chemicals was also suppressed by treatment of cells with haemin. These findings indicate that the level of ALA synthase in HepG2 cells is maintained by both synthesis and degradation of the enzyme, and that the synthesis of the enzyme is regulated by the concentration of regulatory free haem in the cell.

INTRODUCTION

δ-Aminolaevulinate (ALA) synthase [succinyl-CoA: glycine *C*-succinyl transferase (decarboxylating) (EC 2.3.1.37)] is the first enzyme of the haem-biosynthetic pathway and catalyses the condensation of glycine and succinyl-CoA to form ALA. ALA synthase activity in the normal liver is very low, and it is rate-limiting for haem formation [1]. ALA synthase activity in other non-hepatic tissue is generally not inducible by treatment of animals with porphyrogenic chemicals [2], and the non-hepatic enzyme activity is usually not suppressed by treatment with haemin, i.e. Fe^{3+}-protoporphyrin IX [3]. It is not clear, however, whether ALA synthase in uninduced hepatic cells, including the human liver, is controlled by the levels of haem in the cell. If ALA synthase could be studied in isolated human liver cells, it would be very useful for understanding the regulation of haem biosynthesis in human liver and the abnormal metabolic and clinical consequences which are associated with the genetically determined or chemically induced hepatic porphyrias. To our knowledge, no studies have been reported on ALA synthase in isolated liver cells of human origin.

HepG2 cells, a recently described human hepatoma cell line, are morphologically similar to liver parenchymal cells and synthesize major plasma proteins and receptors for a variety of hormones and growth factors [4]. In the present paper, we report that treatment of HepG2 cells with succinylacetone, 3-methylcholanthrene and dimethyl sulphoxide (Me_2SO) results in significant increases of ALA synthase activity, and that haemin and, to a lesser extent, other iron porphyrins and metalloprotoporphyrins repress ALA synthase activity.

MATERIALS AND METHODS

Materials

Me_2SO (spectro grade) was purchased from Fisher, Fair Lawn, NJ, U.S.A.; all tissue-culture materials were from GIBCO, Grand Island, NY, U.S.A. Polybenzimidazole Aurorez resin was provided by Hoechst Celanese, Charlotte, NC, U.S.A. Dowex AG50W-X8 resin (H^+ form, 200–400 mesh) was obtained from Bio-rad, Richmond, CA, U.S.A. [2,3-^{14}C]Succinic acid (40–53 mCi/mmol) was obtained from Amersham, Arlington Heights, IL, U.S.A. Other reagents and chemicals were of reagent grade and obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. $^{59}FeCl_3$ (48 mCi/mg of Fe) was purchased from New England Nuclear, Boston, MA, U.S.A. Fe-deuteroporphyrin, Fe-mesoporphyrin and metalloprotoporphyrins were purchased from Porphyrin Products, Logan, UT, U.S.A.

Cell culture

HepG2 cells were kindly provided by Dr. Barbara Knowles of the Wistar Institute, Philadelphia, PA, U.S.A. Cells were routinely grown in Corning 100 mm × 20 mm tissue-culture dishes (Corning, NY, U.S.A.), in minimum essential medium with Earle's salts supplemented with 10% (v/v) fetal-bovine serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 2 mM-glutamine. Routine subcultures were made weekly at 1:3 dilution, which maintained cells in the exponential growth phase; cells were fed with fresh medium every 2–3 days. Additions of chemicals, e.g. Me_2SO or metalloporphyrins, were made directly to the flasks. Cultures were incubated in the dark, and cells were

Abbreviations used: ALA, δ-aminolaevulinate; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; Me_2SO, dimethyl sulphoxide.

harvested by treatment with 0.05% (w/v) trypsin containing 0.53 mM-EDTA for 5 min at 37 °C, washed and resuspended in Earle's salts and, after 15 passages through a pipette to break up clumps, their numbers were counted in a model F_N Coulter counter.

Preparation of chemical solutions

Haemin, iron porphyrins and metalloprotoporphyrins were prepared at a concentration of 20 mM in 0.2 M-KOH/100% (v/v) methanol (1:1, v/v), and diluted 10-fold with culture medium, as described previously [5]. The solutions were then filtered through a Millipore membrane (0.22 μm pore) for sterilization. Steroids were dissolved in propylene glycol at a concentration of 10 mg/ml. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) and 3-methylcholanthrene were dissolved in Me_2SO at concentrations of 2 mg/ml and 2 mM respectively. $FeCl_3$ (both radiolabelled and unlabelled) was diluted in non-supplemented calf serum (HyClone Laboratories, Logan, UT, U.S.A.), incubated for 30 min at room temperature and stored at 4 °C until use. $^{59}FeCl_3$ was added at a concentration of 1 μCi/ml. Other chemicals were dissolved in sterile water at concentrations 100-fold greater or more than the final concentration in the culture medium.

ALA synthase assay

ALA synthase activity of HepG2 cells was determined by using a radiochemical assay described by Brooker *et al.* [6], with some modifications. The major modification in the procedure was the use of polybenzimidazole resin, instead of ethyl acetate extraction, for rapid and easy recoveries of 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole. The recovery of ALA from the Dowex AG50W-X8 column was 96%. The recovery of the pyrrole was 82% when using the polybenzimidazole resin. Thus, by using Dowex AG50-X8 and polybenzimidazole, the overall recovery of ALA was 79%, and this value was used to correct for the recovery of the product. Although ethyl acetate extracts require concentration before liquid-scintillation counting, eluates from polybenzimidazole columns could be used for counting without concentration.

Cells collected from a 100 mm dish were resuspended in 90 μl of a mixture containing 10 μM-pyridoxal 5'-phosphate, 0.1 mM-(CaMg)EDTA, 0.1% (w/v) Triton X-100, and 5 mM-Tris/HCl, pH 7.4. A 90 μl sample was added to a glass tube (12 mm × 75 mm) for ALA synthase assay, and another sample of 20 μl was used for protein assay. The final assay mixture contained, in a volume of 150 μl, 1.1 mM-sodium succinate including 4 μCi of [2,3-^{14}C]succinic acid/ml, 88.8 mM-glycine, 1 mM-CoA, 2.0 units of succinyl-CoA synthetase (GDP-forming)/ml (0.25 unit/ml, Sigma), 89 μM-GTP, 0.25 mM-pyridoxal 5'-phosphate, 0.89 mM-(CaMg)EDTA, 24.5 mM-sodium laevulinate, 17.8 mM-$MgCl_2$, 222 mM-NaCl, 0.89 mM-dithiothreitol, 50 mM-Tris/HCl, pH 7.4, and 2–4 mg of HepG2 cell proteins. The assay mixture was incubated at 37 °C for 60 min in a water-bath shaker. The reaction was terminated by adding 100 μl of 10%(w/v) trichloroacetic acid, 15 μl of 10 mM-ALA and 15 μl of 1 M-sodium succinate, both dissolved in 1 M-acetate buffer, pH 4.6. After centrifugation at 2500 rev./min for 10 min, the supernatant fraction was saved, and the pellet was treated again with 0.5 ml of 5% trichloroacetic acid. The pH of the pooled supernatant fractions was adjusted to 3.9 with 1 M-sodium acetate. The pH-adjusted solution was placed on a Dowex AG50W-X8 ion-exchange column (2.5 cm × 0.7 cm, equilibrated with 0.1 M-sodium acetate buffer, pH 3.9) and sequentially washed with 5 ml of 0.1 M-sodium acetate buffer, pH 3.9, 5 ml of methanol/0.1 M-sodium acetate buffer, pH 3.9 (2:1, v/v) and 5 ml of 10 mM-HCl. ALA was eluted with 5 ml of 1 M-sodium acetate buffer, pH 8.5, into a glass tube (15 mm × 100 mm) containing 130 μl of 10 M-HCl. ALA was converted into 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole by incubation with 0.2 ml of acetylacetone at 80 °C for 15 min. After cooling, the solution was applied to a column packed with polybenzimidazole resin (2.5 cm × 0.7 cm), equilibrated with 5 mM-sodium acetate buffer, pH 5.0. The column was washed with 5 ml of 5 mM-sodium acetate buffer, pH 5.0, and 5 ml of 0.5 M-formic acid. ALA-pyrrole was eluted into a scintillation vial with 5 ml of ethanol. Then 15 ml of Hydrofluor (National Diagnostics, Manville, NJ, U.S.A.) containing 1% (v/v) acetic acid was added to each vial for liquid-scintillation counting in a Packard Tri-Carb counter. Efficiency of counting was over 85%. Enzyme activity was expressed as pmol of ALA formed/h per mg of protein.

Other assays

ALA dehydratase activity was determined by using 10^6 cells per assay, in accordance with the method described previously [7]. Haem content was determined fluorimetrically by using 10^5 cells per assay [7]. ^{59}Fe incorporation into haem was determined by using 10^6 cells in accordance with the method described previously [5]. Protein concentrations were determined by the method of Lowry *et al.* [8], after digestion of cells in 1 M-NaOH and dilution to 2 ml so that the concentration of Triton (< 0.0001%) did not interefere with the assay. Data were expressed on the basis of mg of protein per assay. Statistical analyses of data were performed by using Student's unpaired one-tail *t* test.

RESULTS

Basal ALA synthase activity

ALA synthase activity in untreated HepG2 cells is shown in Fig. 1. The enzyme activity showed a gradual decline with cell growth in culture, approaching the lowest value of 7.39 ± 0.67 pmol of ALA/h per mg of protein on day 5, which was approximately one-third of the maximal activity found for 3 days after seeding cells in culture. Upon trypsin treatment and seeding cells in culture on day 7, the maximal activity of 25.49 ± 2.96 pmol/h per mg of protein was re-established and maintained for 3 days. Replacing culture medium was partially effective in maintaining the enzyme activity for a further 24 h; however, this was followed by a rapid decline in the enzyme activity thereafter.

Haemin concentrations and ALA synthase activity

Haemin, up to a concentration of 100 μM, had no effect on ALA synthase activity *in vitro* (results not shown). In contrast, ALA synthase activity in HepG2 cells treated for 6 h with haemin in culture showed a dose-dependent decrease in enzyme activity. A half-maximal inhibitory effect of haemin was observed at a concentration of 1 μM (results not shown).

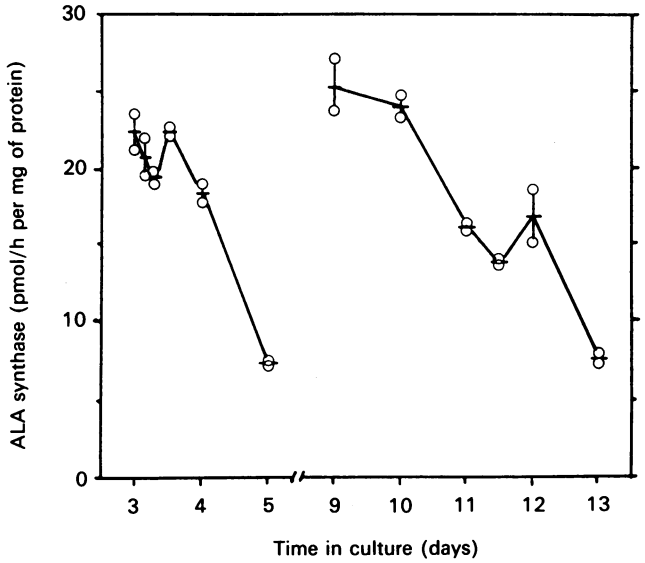

Fig. 1. ALA synthase activity in HepG2 cells as a function of the incubation period

ALA synthase activity was determined in HepG2 cells as a function of the incubation period of cells in culture. Cells were seeded weekly (on days 0 and 7), and fed with fresh media on days 3, 9 and 11. ALA activity was assayed as described in the Materials and methods section. Each circle represents data from each dish, and a horizontal bar represents the mean of duplicate determinations.

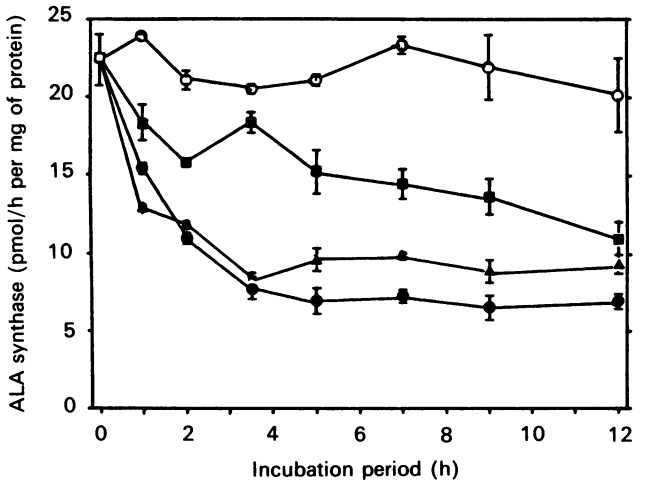

Fig. 2. Time course of ALA synthase activity in HepG2 cells treated with haemin, cycloheximide and actinomycin D

Culture media were replenished 24 h before addition of chemicals. Haemin (10 μM), cycloheximide (1 μg/ml) or actinomycin D (0.25 μg/ml) was added to cultures at zero time. At each time point, cells were harvested for ALA synthase assay. ○, Control; ●, haemin; ▲, cycloheximide; ■, actinomycin D. Data are means ± S.D. of two to three dishes.

Half-lives of ALA synthase

When 10 μM-haemin was added to HepG2 cells, the enzyme activity showed a rapid decline, with a half-life of 2 h (Fig. 2). The half-life of ALA synthase in cells treated with cycloheximide was also 2 h, whereas that of the enzyme in cells treated with actinomycin D was considerably longer (4 h) for the first 4 h than those observed in cells treated with haemin or cycloheximide (Fig. 2).

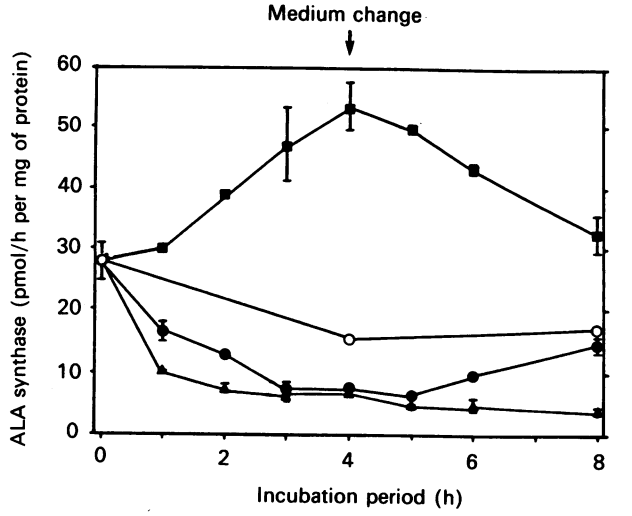

Fig. 3. Time course of ALA synthase activity in HepG2 cells treated with haemin, ALA/iron and succinylacetone

Culture media were replenished 24 h before addition of chemicals. Haemin (100 μM), ALA (1 mM)/$FeCl_3$ (2 μM) or succinylacetone (0.5 mM) was added to cultures at zero time. Culture media were removed at 4 h, and cells were rinsed 3 times with Ca,Mg-free phosphate-buffered saline, and incubated for 4 h in fresh media. At each time point, cells were harvested for ALA synthase assay. ○, Control; ●, haemin; ▲, ALA/$FeCl_3$; ■, succinylacetone. Data are means ± S.D. of two to three dishes.

Time course of ALA synthase activity after treatment with haemin, ALA and iron, or succinylacetone

When cells were treated with 100 μM-haemin for 4 h, and the medium was then replaced with a fresh medium without haemin, ALA synthase activity showed a recovery toward the pretreatment value, and reached 85% of this value 4 h after the change of medium. Since exogenously added haemin was able to decrease ALA synthase activity, we examined the effect of the combined addition of ALA and $FeCl_3$, additions that would increase endogenous haem synthesis. As shown in Fig. 3, the addition of 1 mM-ALA and 2 μM-$FeCl_3$ also strongly suppressed the ALA synthase activity, with the half-life of the enzyme being less than 2 h. Fe^{3+} alone (60 μM-ferric citrate) was not effective in suppressing ALA synthase activity (results not shown). ALA (1 mM) treatment alone resulted in a large amount of porphyrin accumulation, and cells were markedly damaged, as judged from their morphology. When cells were treated with 1 mM-ALA and 2 μM-$FeCl_3$ for 2 days, cells were morphologically intact, and accumulated significantly greater amounts of haem (90.2 ± 3.6 pmol/mg of protein; mean ± S.D., $n = 3$) than did untreated control cells (44.1 ± 6.0 pmol/mg of protein, $n = 3$). The ALA/Fe-treated cells also accumulated significant amounts of porphyrins, i.e. 62.0 ± 5.2 pmol/mg of protein ($n = 3$), which were undetectable in untreated cells. Cells treated with ALA alone accumulated greater amounts of porphyrins than did ALA/Fe-treated cells by at least

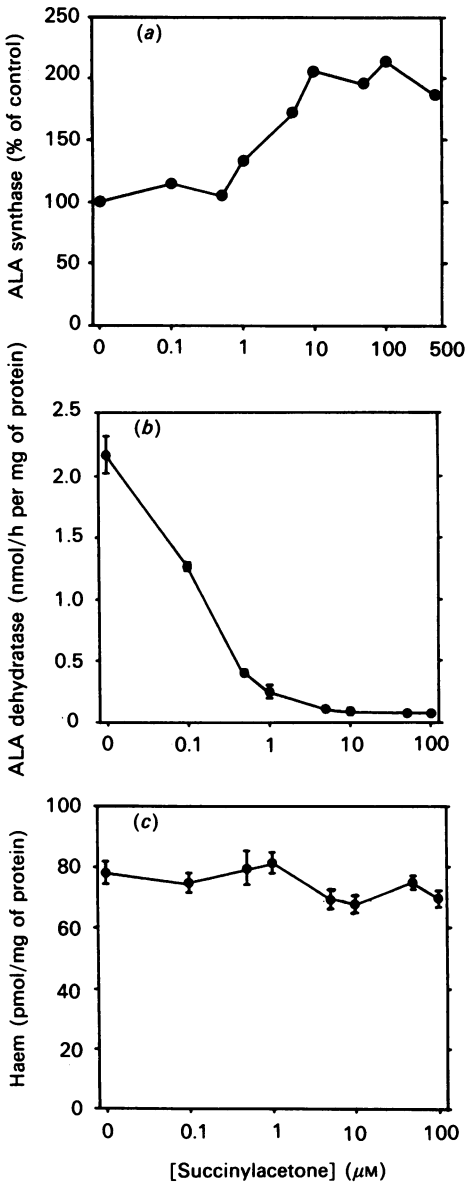

Fig. 4. Effect of succinylacetone on ALA synthase activity, ALA dehydratase activity and haem content of HepG2 cells

HepG2 cells were incubated with various concentrations of succinylacetone for 6 h, and harvested for each assay, as described in the Materials and methods section. (*a*) ALA synthase activity: data are means of two experiments. (*b*) ALA dehydratase activity: data are means ± S.D. of three dishes. (*c*) Haem content: data are means ± S.D. of three dishes.

25%, but accurate determination of porphyrins in the former was not possible after 48 h of incubation, because of cell lysis. At this time, ALA synthase in cells treated with ALA and $FeCl_3$ was markedly decreased (1.27 ± 0.15 pmol/h per mg of protein, $n = 3$) as compared with the untreated controls (10.3 ± 1.40 pmol of ALA/h per mg of protein, $n = 3$).

When cells were treated with ALA and $FeCl_3$ for 4 h and the medium was then replaced with fresh medium without these chemicals, ALA synthase activity remained suppressed for the subsequent 4 h (Fig. 3). Thus the effect of the haem endogenously synthesized from ALA and $FeCl_3$ in suppressing ALA synthase activity was substantially prolonged over that produced by the addition of exogenous haemin.

In order to determine whether haem deficiency derepresses ALA synthase activity, HepG2 cells were treated for 4 h with succinylacetone, a potent inhibitor of ALA dehydratase [9]. ALA synthase activity was increased in cells starting 2 h after the addition of succinylacetone, and reached a maximum at 4 h (Fig. 3). When the medium was replaced with fresh medium not containing succinylacetone, ALA synthase activity gradually declined and returned almost to the normal value 4 h after replenishment of the medium (Fig. 3).

Effects of succinlyacetone on ALA synthase, ALA dehydratase and haem content

HepG2 cells were treated for 6 h with succinylacetone, and its effects on ALA synthase activity were examined as a function of succinylactone concentration. In addition, ALA dehydratase activity and haem content were determined. Succinylacetone treatment produced a dose-dependent increase in ALA synthase activity, starting at 5 μM, and a maximal increase in the enzyme activity was observe at 10 μM (Fig. 4*a*). ALA dehydratase activity was strongly inhibited by succinylacetone. For example, the enzyme activity was inhibited by 90% at 1 μM-succinylacetone, and the maximal inhibition was observed at 10 μM (Fig. 4*b*). Although succinylacetone strongly inhibited ALA dehydratase activity, it had little effect on haem concentration in HepG2 cells (Fig. 4*c*).

Effects of ALA and succinylacetone on the incorporation of ^{59}Fe into haem

HepG2 cells were incubated with ^{59}Fe (1 μCi/ml; 1.2 μM) and the effects of the addition of ALA, succinylacetone or haemin on the incorporation of ^{59}Fe into haem were examined. Cellular uptake of ^{59}Fe (0.3% of the added iron) was not affected by ALA, succinylacetone or haemin treatment. However, ^{59}Fe incorporation into the haem fraction was significantly increased by ALA treatment, whereas it was essentially abolished by succinylacetone or haemin treatment (Table 1). The rates of ^{59}Fe utilization for haem synthesis, calculated as the amount of ^{59}Fe utilized for haem synthesis as a fraction of the total cellular ^{59}Fe, are shown in the third column of Table 1. These findings show that ALA treatment significantly increased haem synthesis, whereas succinylacetone or haemin treatment markedly diminished it.

Effects of other Fe-porphyrins and metalloprotoporphyrins on ALA synthase activity

In order to determine the specificty of haemin with respect to the induction of ALA synthase activity, we examined the effects of two other Fe-porphyrins, i.e. Fe-deuteroporphyrin and Fe-mesoporphyrin, and four metalloprotoporphyrins, i.e. Co-, Mn-, Sn- and Zn-protoporphyrin, on this enzyme activity. Since the maximal inhibition of ALA synthase with haemin was observed 6 h after the initiation of incubation (Fig. 2), cells were incubated with these porphyrins for 6 h, and ALA synthase activity was then determined. Although Fe-deuteroporphyrin and Fe-mesoporphyrin also inhibited ALA synthase activity, their effects were considerably weaker than that of haemin (results not shown), namely that the concentrations which resulted in a half-maximal inhibition were 10 μM and 100 μM for Fe-

Table 1. Effects of ALA, succinylacetone and haemin on the incorporation of ^{59}Fe into haem of HepG2 cells

HepG2 cells were incubated for 48 h with 1 μCi of $^{59}FeCl_3$/ml (1.2 μM) in the presence or absence of 1 mM-ALA, 0.5 mM-succinylacetone or 100 μM-haemin. After harvesting of cells, haem was extracted, and radioactivity in cells and in the haem fraction was counted as described in the Materials and methods section. Data are means ± S.D. for three dishes: ns, not significant, * $P < 0.005$, ** $P < 0.001$ versus control in the same experiment.

Treatment	^{59}Fe (c.p.m./mg of cell protein): in cells	in haem	Percentage of cellular ^{59}Fe used for haem synthesis
Control	23928 ± 2743	959 ± 33	4.03 ± 0.33
ALA	22516 ± 2114 ns	2271 ± 171*	10.10 ± 0.20**
Succinylacetone	20616 ± 775 ns	53 ± 12**	0.26 ± 0.06**
Haemin	24019 ± 528 ns	20 ± 9**	0.08 ± 0.04**

deuteroporphyrin and Fe-mesoporphyrin respectively, as compared with 1 μM for Fe-protoporphyrin. Co-, Mn-, Sn- and Zn-protoporphyrin also inhibited ALA synthase activity at high concentrations, i.e. 10 μM and 100 μM, whereas none of them produced enzyme inhibition at 1 μM as did haemin (results not shown).

Effects of porphyrogenic chemicals on ALA synthase activity

Effects of chemicals which are porphyrogenic in chick-embryo hepatocyte cultures were examined by treating HepG2 cells with the chemical for 0, 3, 6, 12, 24 and 48 h, after which ALA synthase activity was determined. DDC (10 μg/ml), phenobarbital (100 μg/ml), 3α-hydroxy-5α-androstan-17-one (androsterone) (5 μg/ml) and 3α-hydroxy-5β-androstan-17-one (etiocholanolone) (5 μg/ml), used at concentrations known to elicit porphyrogenic responses in chick-embryo hepatocyte cultures [10], did not significantly affect ALA synthase activity at any time point (results not shown). The combined addition of these porphyrogenic chemicals and succinylacetone at the concentration (1 μM) which inhibits ALA dehydratase significantly but does not affect ALA synthase (Figs. 4*b* and 4*a*) also did not affect ALA synthase activity in HepG2 cells (results not shown).

Effects of 3-methylcholanthrene on ALA synthase activity

3-Methylcholanthrene (1 μM) had no effect on ALA synthase activity up to 24 h, but it increased ALA synthase activity thereafter (results not shown). Effects of 3-methylcholanthrene on enzyme activity were dose-dependent up to 1 μM (Fig. 5), but the chemical became toxic to the cells at higher concentrations, as judged from alterations of cell morphology and loss of protein. At the highest concentration of 3-methylcholanthrene i.e. 2.5 μM, the final concentration of Me_2SO used as vehicle was 0.125% (v/v), which by itself had no effect on ALA synthase activity (results not shown).

Effects of Me_2SO on ALA synthase activity

Me_2SO has been shown to stimulate haem synthesis and the synthesis of ALA dehydratase *de novo* in HepG2 cells [11,12]. Treatment of HepG2 cells with 2% Me_2SO for 72 h produced a significant increase in ALA synthase activity (15.13 ± 0.27 pmol of ALA/h per mg of protein; mean ± S.D., $n = 3$) compared with the untreated control (7.62 ± 0.29 pmol/h per mg; $n = 3$; $P < 0.001$). Combined addition of 2% Me_2SO and 1 μM-3-methylcholanthrene resulted in a higher enzyme activity (19.16 ± 1.81 pmol of ALA/h per mg of protein; $n = 3$; $P < 0.05$ versus Me_2SO-treated cells).

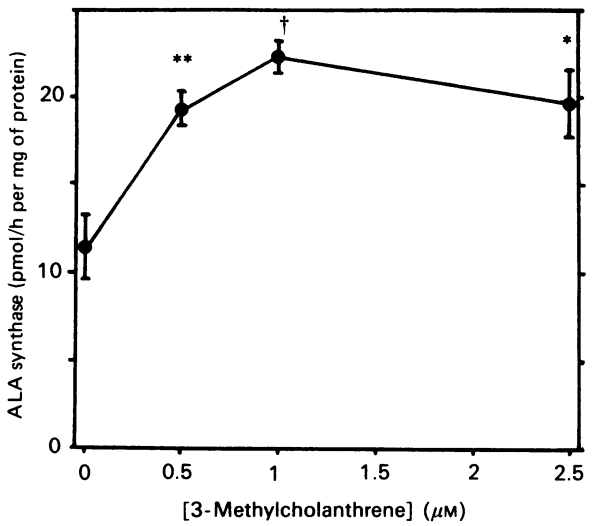

Fig. 5. Effect of 3-methylcholanthrene on ALA synthase activity

Cells were treated with 3-methylcholanthrene at concentrations indicated on the abscissa for 72 h. Culture media were replenished with the chemical at 48 h. ALA synthase activity was determined as described in the Materials and methods section. Data are means ± S.D. of three dishes: ns, not significant: * $P < 0.01$; ** $P < 0.005$; † $P < 0.001$ versus untreated control.

Effects of haemin on the increased ALA synthase activity mediated by succinylacetone, 3-methylcholanthrene and Me_2SO

The increases in ALA synthase activity mediated by succinylacetone, 3-methylcholanthrene and Me_2SO were all suppressed by simultaneous treatment of cells with 10 μM-haemin (Table 2). In addition, when haemin was added only for the last 6 h of the total incubation period of 72 h, the enzyme activity could still be suppressed by haemin treatment.

Table 2. Effects of haemin on increased ALA synthase activity mediated by succinylacetone, 3-methylcholanthrene and Me_2SO in HepG2 cells

HepG2 cells were incubated with 100 μM-succinylacetone, 1 μM-3-methylcholanthrene or 2% Me_2SO for 72 h. Haemin (10 μM) was added to the culture either at 0 h or at 66 h. Media were replenished with chemicals at 48 h. Total incubation periods after the seeding of cells in culture were 6 days and 5 days for Expts. 1 and 2 respectively. Data are the means ± S.D. of three dishes: ns, not significant; † $P < 0.01$; * $P < 0.005$; ** $P < 0.001$ versus control in the same experiment.

Treatment	Haemin treatment...	ALA synthase activity (pmol of ALA/h per mg of protein) None	0–72 h	66–72 h
Expt. 1.				
Control		8.29 ± 0.79	2.66 ± 0.22	2.62 ± 0.40
Succinylacetone		32.30 ± 1.44**	3.51 ± 0.69 ns	7.68 ± 0.95*
3-Methylcholanthrene		13.81 ± 1.77‡	3.99 ± 0.19*	7.45 ± 0.83**
Expt. 2				
Control		14.54 ± 0.39	6.81 ± 2.15	4.79 ± 1.54
Me_2SO		21.09 ± 1.03*	10.04 ± 1.39 ns	6.82 ± 0.39 ns

DISCUSSION

We have reported previously that HepG2 cells retain measurable activities of ALA dehydratase [11], porphobilinogen deaminase [12], uroporphyrinogen decarboxylase [12], haem content [11], cytochrome *P*-450 [13] and mixed-function oxidase activities [13]. These findings are of considerable significance, since ALA dehydratase activity is known to show a precipitous decline upon isolation of rat liver cells for viable non-proliferating cultures [14]. Treatment of cells with Me_2SO results in an increased synthesis of immunochemically quantifiable ALA dehydratase, and increased haem content [11,12], whereas porphobilinogen deaminase and uroporphyrinogen decarboxylase activities are decreased. Our findings in the present study indicate that ALA synthase activity can be demonstrated in HepG2 cells, and that ALA synthase activity decreases as a function of incubation period of cells in culture, but is restored to its normal level when cells are trypsin treated and re-seeded in culture. This is an important finding in that, by contrast, ALA synthase activity in primary cultures of chick-embryo hepatocytes, in which the drug-mediated induction of ALA synthase can be demonstrated up to 4 days [15], becomes undetectable after 7 days in culture [16].

ALA synthase activity in HepG2 cells is strongly inhibited by exogenously administered haemin at 1 μM, but is affected much less by two other iron-porphyrins, i.e. Fe-meso- and Fe-deutero-porphyrin, and by four metalloprotoporphyrins, i.e. Co-, Mn-, Sn- and Zn-protoporphyrin (results not shown). Although all of these prophyrins inhibited ALA synthase activity at 10 μM or higher concentrations, only haemin was significantly inhibitory at 1 μM. These data suggest that the formation of ALA synthase is inhibited specifically and most potently by haem, the end-product of the biosynthetic pathway, for which it is the rate-limiting enzyme. This point has been substantiated by the fact that treatment of cells with ALA and $FeCl_3$, which increases endogenous haem synthesis as evidenced by increased ^{59}Fe incorporation into haem (Table 1), significantly inhibited ALA synthase activity and decreased its half-life (Fig. 3). Our finding also suggests that ALA treatment alone led to an over-load of precursors to the ferrochelatase step, resulting in excessive accumulation of porphyrins, whereas combined treatment of cells with ALA and $FeCl_3$ permitted haem synthesis, thereby eliminating the porphyrin-mediated toxicity to the cell. Thus, in these cultures, iron appears to be required for maximal synthesis of haem, even when an excess of ALA or haem precursors is available. Similar effects of ALA and iron have been reported in other cell types [17–20], including human lymphocytes [17–19].

Our findings in this paper are compatible with the regulation of ALA synthase formation at the level of its synthesis and degradation. The inhibitory effect on ALA synthase formation by ALA and iron occurred more rapidly, achieved a greater level of suppression, and lasted longer than that produced by haemin (Fig. 3). Presumably the difference observed between the effects of haemin and the combined addition of ALA and $FeCl_3$ are due to differences in the concentration of regulatory haem achieved by each treatment. It is possible that the combined ALA/$FeCl_3$ treatment attained a greater concentration of regulatory free haem than with 100 μM-haemin because cells are more permeable to them than to haemin, or haem may be continuously synthesized in the cell from haem precursors, e.g. porphobilinogen or porphyrinogens, even after ALA and iron were washed out. The effect of haemin on the increase in the concentration of regulatory free haem may also be compounded by the fact that treatment of cells with haemin markedly decreases endogenous haem synthesis from iron (Table 1).

Treatment of cells with actinomycin D resulted in a longer half-life of the enzyme, i.e. 4 h, than did treatment with cycloheximide or haemin (Fig. 2). This finding suggests that the half-life of the mRNA for ALA synthase is considerably longer than that of ALA synthase itself, and that the inhibitory action of haemin is exerted at a more post-transcriptional than transcriptional step on the process of enzyme synthesis. This finding appears at variance with the finding in the rat liver in which the half-life of ALA synthase mRNA after the injection of

α-amanitin was found to be 20 min [21]. In cultured chick-embryo liver cells treated with 2-allyl-2-isopropylacetamide or the 5β-steroid etiocholanolone, the half-life of ALA synthase after acetoxycycloheximide treatment was 3 h, and the apparent half-life of the enzyme after actinomycin D treatment was 5.2 h, which included both the decay of mRNA and of the enzyme [22].

Although succinylacetone inhibited ALA dehydratase activity (Fig. 4*b*) and derepressed ALA synthase activity (Fig. 4*a*), it had little effect on the total cellular haem content (Fig. 4*c*). On the other hand, it is clear from our findings that succinylacetone abolished haem synthesis, as determined by ^{59}Fe incorporation into haem (Table 1), and that ALA synthase activity is influenced by intracellular regulatory haem concentrations, as shown in the case of treatment with haemin or ALA/iron (Figs. 2 and 3). These findings suggest that the regulatory haem pool largely reflects newly synthesized haem, but not total cellular haem content.

Porphyrogenic chemicals in chick embryos such as DDC [23], 5α- or 5β-steroids [10,24] or phenobarbital [25] failed to increase ALA synthase activity in HepG2 cells. Although succinylacetone treatment is known to enhance greatly the induction response of chick-embryo hepatocytes to such porphyrogenic chemicals [9], the combined addition of succinylacetone with DDC, androsterone, etiocholanolone or phenobarbital did not affect ALA synthase activity. On the other hand, 3-methylcholanthrene, a potent inducer of certain cytochrome *P*-450 isoenzymes [26], increased ALA synthase activity in a dose-dependent manner, reaching a maximal increase of 2-fold at 1 μM (Fig. 5). The extent of ALA synthase induction by 3-methylcholanthrene in HepG2 cells was similar to that in rat liver *in vivo*, the increase in ALA synthase observed in rat liver after 3-methylcholanthrene treatment being approx. 45% over the untreated control value [27]. The chemical-mediated induction of ALA synthase in HepG2 cells was also shown to be subject to suppression by haemin (Table 2). These findings indicate that HepG2 cells retain the ability to respond to limited numbers of chemicals, such as succinylacetone, 3-methylcholanthrene and Me_2SO, by displaying an induction response of ALA synthase, and that both the non-induced and the induced ALA synthase activities are subject to haemin suppression. This finding is of considerable significance, since it is known that ALA synthase is no longer inducible after 3 days in cultured chick-embryo liver cells [16].

This is the first extensive study on ALA synthase in human liver-derived cells. The rapid turnover of ALA synthase in untreated cells, the repression of enzyme synthesis by haem, the derepression by succinylacetone, and the induction by 3-methylcholanthrene and Me_2SO of ALA synthase in HepG2 cells, are new findings in this cell type. Our data also clearly indicate that the regulatory free haem pool controlling the synthesis of ALA synthase is a fraction of newly synthesized haem, and not the total content of cellular haem. These findings suggest that HepG2 cells may be a useful model for the study of the control of haem synthesis in human liver-derived cells.

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