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ORGAN TOXICITY AND MECHANISMS

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Inducing coproporphyria in rat hepatocyte cultures using cyclic AMP and cyclic AMP-releasing agents

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Abstract Cyclic AMP (c-AMP), added on its own to rat hepatocyte cultures, caused a marked accumulation of coproporphyrin III. The results obtained by comparing the effect of c-AMP to that of exogenous 5-aminolevulinate (ALA), and from adding c-AMP and ALA together, indicated that the coproporphyrinogen III metabolism was blocked, even though no inhibition of the relevant enzyme, coproporphyrinogen oxidase, could be demonstrated. Preferential accumulation of coproporphyrin could also be produced in cultures of rat hepatocytes by agents that raise the cellular levels of cyclic AMP, such as glucagon. The effect of supplementing the culture medium with triiodothyronine (T3) on the response of rat hepatocytes to c-AMP was also investigated. T3, which is known to stimulate mitochondrial respiration, uncoupling O_2 consumption from ATP synthesis, produced a c-AMP-like effect when given on its own and potentiated the effect of c-AMP, with an apparent increase in the severity of the metabolic block. It is suggested that an oxidative mechanism may be activated in c-AMP and T_3-induced coproporphyria, preferentially involving the mitochondrial compartment, leading to oxidation of porphyrinogen intermediates of haem biosynthesis, especially coproporphyrinogen. Coproporphyin, the fully oxidized aromatic derivative produced, cannot be metabolized and will therefore accumulate.

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Introduction

Hepatocytes in primary culture are a sensitive system for detecting drug-induced toxic responses of the liver, including disorders of the pathway of haem biosynthesis, such as hepatic porphyrias. For example, chick embryo hepatocytes are very sensitive to the drug-dependent induction of uroporphyria, a metabolic disorder characterized by a block in uroporphyrinogen metabolism and closely resembling human *porphyria cutanea tarda* (Marks 1986). In contrast to chick embryo hepatocytes, hepatocytes from adult rats do not respond to uroporphyria-inducing drugs in culture (De Matteis et al 1988). Liver microsomes from chicken embryo hepatocytes are also very much more active than liver microsomes from rats (De Matteis et al 2002) in the oxidation of an intermediate of haem biosynthesis, uroporphyrinogen, to its aromatic derivative, uroporphyrin, which cannot be metabolized and will therefore accumulate. This oxidative mechanism, which can be referred to as the "oxidative escape" of porphyrinogens from the pathway of haem biosynthesis, has been proposed by several authors to be an important factor in the induction of uroporphyria (Heikel et al 1958; Sinclair et al 1987; Smith and De Matteis 1990; Smith and Francis 1993).

The addition of cyclic AMP (c-AMP) has been reported (Edwards and Elliott1974) to be essential to the drug-induced stimulation of porphyrin biosynthesis in cultures of rat hepatocytes, but the lack of response of the rat system to uroporphyria-inducing drugs was not overcome by the addition of c-AMP (De Matteis and Harvey 1987). In those studies we found, however, that c-AMP added on its own to cultured rat hepatocytes caused marked accumulation of porphyrins, with coproporphyrin III predominating (De Matteis and Harvey 1987). To our knowledge, this is the first

instance of a porphyrin metabolism disorder induced in a mammalian system by a physiologically-active agent given on its own (in the absence, that is, of a porphyria-inducing drug or of the genetic defect of acute porphyria) and the purpose of the present report is to describe the c-AMP-induced porphyria in detail.

Scheme 1 shows the main steps in the biosynthesis of haem. A compartmentation of the biosynthetic enzymes exists in mammalian liver (Sano and Granick1961), with the first step [formation of 5-aminolevulinate (ALA)] and the last steps (leading to protoporphyrin and haem) taking place within the mitochondrion, while the intermediary biosynthetic steps (concerned with the formation and decarboxylation of uroporphyrinogen) occur in the cytoplasm.

Scheme 1 also shows three possible mechanisms by which c-AMP might induce accumulation of coproporphyrin:(1) c-AMP might inhibit the enzyme coproporphyrinogen oxidase; (2) since coproporphyrinogen has to cross the outer mitochrondrial membrane in order to reach its metabolism site, c-AMP could possibly interfere with any such transport mechanism; (3) finally, it might cause oxidation of coproporphyrinogen to coproporphyrin, which cannot be further metabolized (an oxidative escape mechanism, similar to that discussed above for uroporphyrinogen in hepatic uroporphyria).

In this work we compared the effect of adding c-AMP to that of adding exogenous 5-aminolevulinate (ALA), and examined, in additional experiments, the effect of c-AMP and ALA added together. We also investigated whether accumulation of coproporphyrin III could also be induced in cultures of rat hepatocytes by agents, such as glucagon and isoproterenol, that raise the cellular levels of c-AMP. Finally, we studied the effect of triiodothyronine (T3), which is known to stimulate mitochondrial respiration, uncoupling O_2 consumption from ATP synthesis.

Materials and methods

Sources of special chemicals

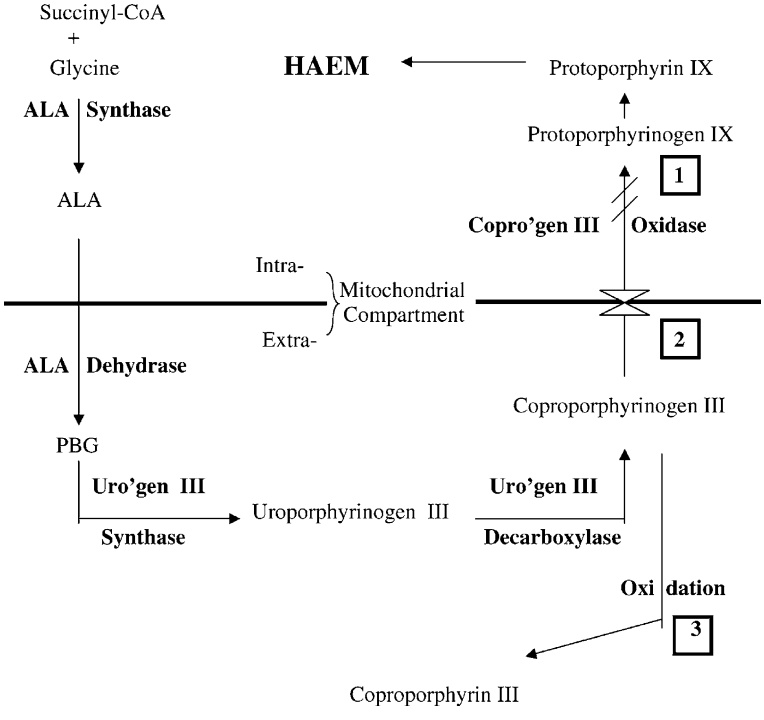
The source of the culture medium and of most of its components has been stated previously (Ferioli et al 1984). Triiodothyronine (T_3), isoproterenol and dibutyryl-adenosine 3′,5′-monophosphate (c-AMP) were obtained from Sigma Chemical Co., Poole, Dorset, UK. Glucagon was from Serva, Heidelberg, Germany. Coproporphyrin III, uroporphyrin III, protoporphyrin and mesoporphyrin were all from Porphyrin Products, Logan, UT, USA.

Animals

Fed male adult rats of the Porton (Wistar-derived) strain (body weight 180–200 g) were used. All experiments were conducted in compliance with the regulations governing animal care in the United Kingdom.

Preparation and treatment of cultures

Rat hepatocytes (viability 81–94%) were obtained by a collagenase perfusion technique (Paine and Legg 1978)



Scheme 1 Main steps in the biosynthesis of haem, showing the compartimentation of the biosynthetic enzymes and possible mechanisms by which c-AMP might induce accumulation of coproporphyrin: 1) inhibition of the enzyme coproporphyrinogen oxidase; 2) interference with any transport mechanism utilized by coproporphyrinogen in order to cross the mitochondrial membrane; 3) oxidation of coproporphyrinogen to coproporphyrin (the latter may take place preferentially within the mitochondrion, see text). ALA: 5-aminolevulinic acid; PBG: porphobilinogen

and were cultured (0.5×10^6 cells/mL of culture medium) in eight-well multi-plates (2 mL/well), as described previously (Ferioli et al 1984). A change to serum-free medium was carried out at 20 h and again at 22 h; drugs and/or ALA were added 24 h after seeding and the cultures were examined 2–72 h (usually 19 h) later. In the experiments aimed at measuring the activity of ALA-synthase or coproporphyrinogen oxidase in vitro, rat hepatocytes were similarly cultured in 100 mm diameter dishes (10 mL/dish).

Analytical techniques

Porphyrins were assayed in cell extracts and in media as described (Ferioli et al 1984) using a fluorimetric method (Grandchamp et al 1980). The identity of the accumulating porphyrin was also established by a reverse-phase gradient HPLC technique (De Matteis et al 1987). Cellular proteins were measured by the method of Lowry et al (1951), after solubilization of the acid-extracted monolayers in alkali.

Enzyme assays

The activity of coproporphyrinogen III oxidase was determined with either freshly obtained or cultured hepatocytes, using the whole cell homogenate which had been frozen and thawed once. The technique described by Grandchamp and Nordmann (1982) was followed, except that unlabelled (rather than [14C]-labelled) coproporphyrinogen was used, the addition of carrier protoporphyrin was omitted and an internal standard of mesoporphyrin (250 nmol in 5 μL DMSO) was added to each sample. After incubating for 1 h at 37 °C in the dark with shaking, the reaction was stopped by adding 0.75 mL ethyl Acetate/acetic acid mixture (4:1, by vol) and the extraction was repeated twice more with 1 mL ethyl acetate. The combined extracts were taken to dryness, redissolved in methanol and subjected to HPLC (Lim and Peters 1988), except that the proportion of methanol and 1 M ammonium acetate in the elution system were adjusted to 88:12, by vol, as this ratio was found to yield a better resolution of mesoporphyrin from protoporphyrin.

The activity of ALA-synthase was assayed using whole cell homogenates (De Matteis et al 1981).

Results and discussion

Cyclic AMP-dependent coproporphyria in rat hepatocytes

We have previously reported (De Matteis and Harvey 1987) that c-AMP added to rat hepatocyte cultures causes a marked accumulation of porphyrins, with coproporphyrin III predominating. Here we study the c-AMP-induced porphyria in detail.

In the first experiment, we compared the effect of c-AMP with that of exogenous ALA, the first intermediate in the pathway of porphyrin biosynthesis. The time course of porphyrin accumulation after adding c-AMP to rat hepatocytes is shown in Fig. 1; and that observed after adding ALA is shown in Fig. 2. Both c-AMP and ALA caused a time-dependent increase in porphyrins, but the compositions of the accumulating porphyrins differed significantly in the two cases. With c-AMP, coproporphyrin clearly predominated, especially at the later time points, when the contribution of protoporphyrin gradually declined and coproporphyrin accounted for over half of the total porphyrin accumulating. In contrast, with ALA protoporphyrin was the predominant porphyrin at all time points, although its percentage contribution declined after 24 h incubation, when a rise in uroporphyrin was noted. Similar cultures

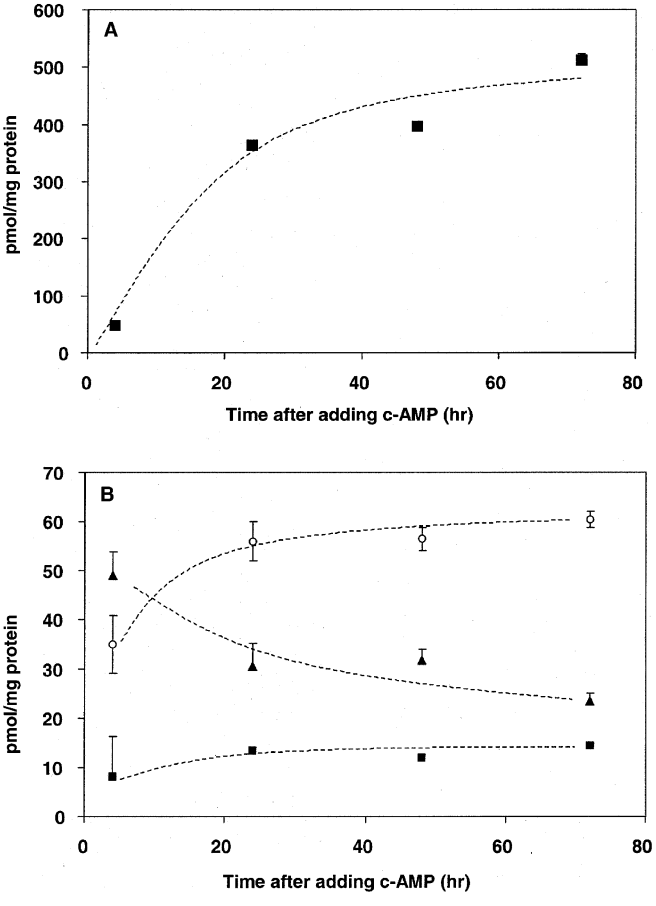


Fig. 1A–B Time course of porphyrin accumulation after adding a single dose of c-AMP (at a final concentration of 50 μM) to rat hepatocyte cultures. Total porphyrins are given in **A** (as pmol/mg protein) and the percentage composition of the accumulating porphyrins in **B**, where they are indicated thus: *triangles*, protoporphyrin; *circles*, coproporphyrin; *squares*, uroporphyrin. Results are averages ± SEM of four observations. Please note that in this and following figures, if error bars are not visible they are contained within the area of the symbols

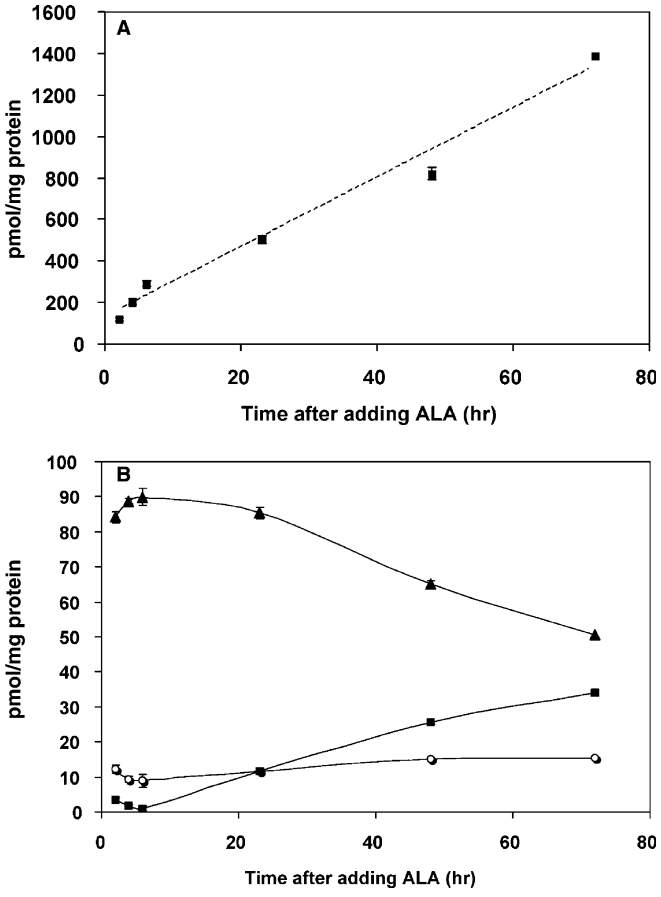


Fig. 2A–B Time course of porphyrin accumulation after adding a single dose of exogenous ALA (final concentration 25 μg/mL) to rat hepatocytes cultures. **A** shows the total porphyrin accumulating (as pmol/mg protein) and **B** its percentage composition, which is indicated thus: *triangles*, protoporphyrin; *circles*, coproporphyrin; *squares*, uroporphyrin. Results are averages ± SEM of four observations

of hepatocytes incubated without any addition showed only a slight increase in porphyrins with time (~1.5-fold), and their porphyrin profile resembled that shown in Fig. 2 for cultures treated with ALA, as here too protoporphyrin predominated. These findings suggested that, in contrast to the normal conditions where ferrochelatase is limiting (hence the accumulation of protoporphyrin), after c-AMP there is a block in the pathway at the level of coproporphyrinogen metabolism. The results also suggest that the effect of c-AMP is unlikely to be due merely to increased production of ALA, through activation or induction of ALA-synthase, a conclusion born out by direct measurament of the synthase activity, which showed only a modest stimulation after treatment. When ALA-synthase was measured using homogenates of rat hepatocytes harvested 19 h after treatment with c-AMP (50 μM) or appropriate controls, the amount (in pMol) of ALA synthesized/min/mg of cellular protein (average with individual observations in brackets) were as follows: control, 2.0 (2.17, 1.85); c-AMP, 2.95 (3.3, 2.6). This finding is also compatible with a partial block in haem biosynthesis, leading to a secondary, compensatory increase in the activity of the synthase.

More direct evidence for the notion that c-AMP causes a block in the metabolism of coproporphyrinogen was obtained by comparing the dose responses to c-AMP in the absence (Fig. 3) and in the presence of ALA (Fig. 4). Figure 3 shows the dose-dependent increase in total porphyrins and in the percent contribution of coproporphyrin, with an EC_{50} of ~3 μM c-AMP for both effects. Figure 4 shows the dose-dependent response to c-AMP in the presence of exogenous ALA, under conditions where the rate-controlling step of the pathway is bypassed, and the supply of porphyrin precursors is therefore maintained as fairly constant. Here again, as the c-AMP was raised, the porphyrin profile gradually changed in favour of coproporphyrin (and, to a much less extent, uroporphyrin) at the expense of protoporphyrin. The coproporphyrin accumulating after treatment with c-AMP, whether in the presence or absence of exogenous ALA, was found almost entirely in the cul-

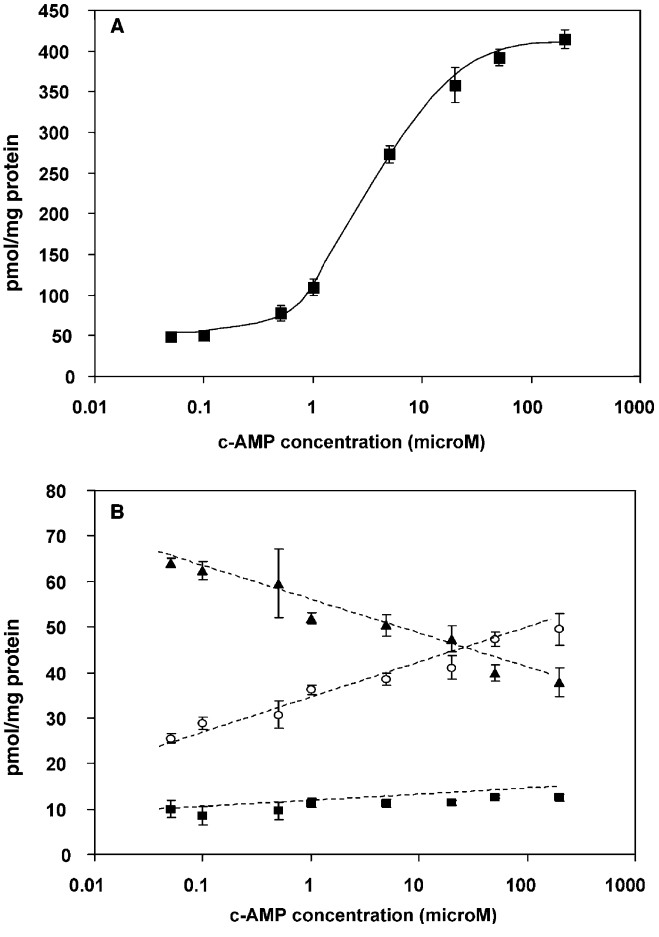


Fig. 3A–B Effect of adding increasing amounts of c-AMP to rat hepatocyte cultures on the amount of porphyrins accumulating in the absence of exogenous ALA. Total porphyrins are given in **A** (as pmol/mg protein). The percentage composition of the accumulating porphyrins is given in **B**and is indicated thus: *triangles*, protoporphyrin; *circles*, coproporphyrin; *squares*, uroporphyrin. Results are averages ± SEM of at least four observations

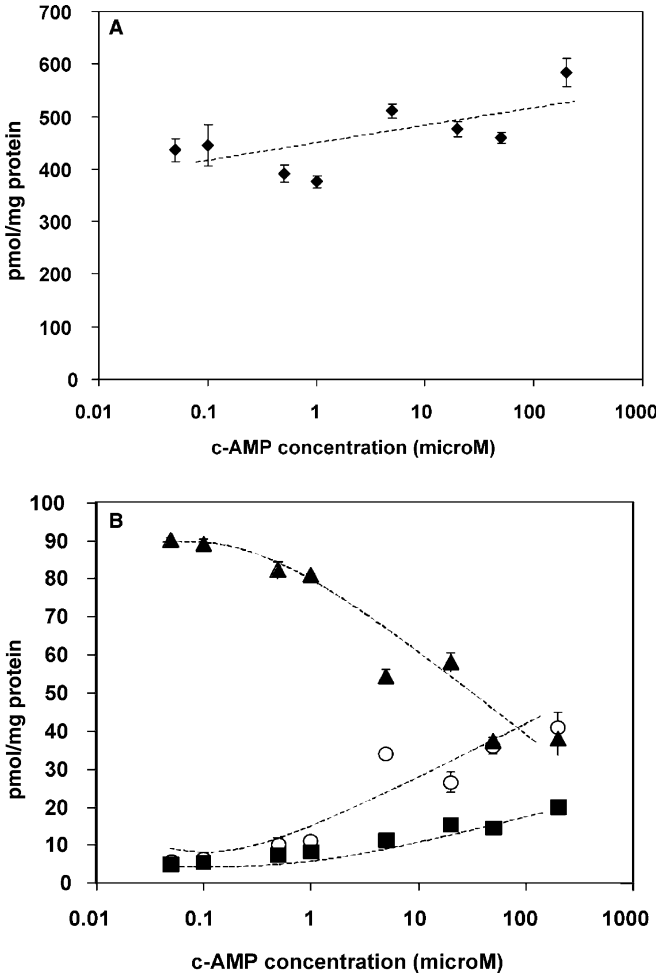


Fig. 4A–B Dose-dependent response to c-AMP in the presence of exogenous ALA. Total porphyrins recovered in the cultures (see legend to Table 1 for the experimental conditions) are given in **A** (as pmol/mg protein) and the percent composition in **B**: *triangles*, protoporphyrin; *circles*, coproporphyrin; *squares*, uroporphyrin. Results are averages ± SEM of at least four observations

ture medium and was identified as the type III isomer by HPLC.

Glucagon-induced and isoproterenol-induced coproporphyria in rat hepatocyte cultures

The effects of glucagon and isoproterenol, two agents that are known to raise the concentration of c-AMP in the liver, were also studied and compared to the effect of c-AMP itself. Both glucagon and isoproterenol resembled c-AMP: (a) by raising the total porphyrin content of the cultures when added on their own (in the absence of exogenous ALA); and (b) by causing preferential accumulation of coproporphyrin from exogenous ALA at the expense of protoporphyrin (Table 1). Here again, although coproporphyrin was the main porphyrin to accumulate, a significant increase in uroporphyrin was also observed in the presence of exogenous ALA, and of both uroporphyrin and protoporphyrin in its absence.

Accumulation of coproporphyrin III may be due to a defect of the mitochondrial enzyme coproporphyrinogen oxidase, as found in genetic coproporphyria, where the activity of this enzyme is characteristically depressed. However, no loss of coproporphyrinogen oxidase activity was found in hepatocytes cultured in the presence of c-AMP (50 μM) for either 3, 5 or 19 h. Enzyme activities of cell homogenates were as follows. Freshly obtained hepatocytes, before culturing: 740 ± 85 pmol protoporphyrin produced/h/mg protein, mean ± SEM of four observations. After culturing (expressed as a percentage of appropriate "fresh hepatocyte" activity, means with individual results in brackets), controls: 3 h, 117% (112, 122); 5 h, 106%; 19 h, 104% (110, 125, 76); c-AMP: 3 h, 114% (96, 133); 5 h, 107%; 19 h, 100% (104, 99, 98).

Since no inhibition of coproporphyrinogen oxidase could be demonstrated, an alternative explanation for the accumulation of coproporphyrin is an "oxidative escape" of coproporphyrinogen from the pathway of haem biosynthesis, since—as pointed out above—coproporphyrin, the fully oxidized aromatic derivative, cannot be metabolized and will therefore accumulate. Some support for this view is offered by recent findings that c-AMP can modulate the activity of mitochondrial electron transport by phosphorylating a 18 kDa subunit of Complex I, leading to stimulation of Complex I activity, increased mitochondrial respiration (Papa 2002; Papa et al 2002; Tsuboi et al 2003) and, potentially, to increased production of oxygen radicals within the mitochondrial compartment (Raha et al 2002). Thyroid hormones are also known to increase mitochondrial respiration and to stimulate production of O_2^- and of H_2O_2 within the mitochondria (Venditti et al 1999; Castilho et al 1998; Fernandez and Videla 1993). They promote uncoupling between oxygen consumption and ATP synthesis (Barbe et al 2001) by increasing transcription of uncoupling protein genes, an effect where interaction with c-AMP has been reported (Rabelo et al 1995). We therefore investigated whether triiodothyronine (T_3) could elicit changes in coproporphyrin excretion in our rat hepatocyte system and interact with c-AMP in this particular response.

Effect of triiodothyronine on the amount of porphyrin that accumulates in cultures of rat hepatocytes

T_3 increased the response to c-AMP both in terms of total porphyrin accumulation in the absence of exogenous ALA and also with respect to the preferential accumulation of coproporphyrin. T3 itself produced a small c-AMP-like effect, but the result of administering T_3 and c-AMP together suggested some degree of potentiation or synergism, especially in the absence of exogenous ALA (Table 2). These effects of T_3 added to rat hepatocyte cultures at the concentrations given in the legend to Table 2 were confirmed three times. In two additional experiments, T_3 was only added as a

Table 1 Effects of glucagon and isoproterenol on the porphyrin contents of rat hepatocyte cultures and on the conversion of exogenous ALA to porphyrins

Additions	Porphyrins of combined cells and medium (pmol/mg protein)			
	Total	Uro	Copro	Proto
		(percent of total given in brackets)		
A) Exogenous ALA absent				
None	87.4 ± 2	8.8 ± 0.2(10.1)	26 ± 1(29.9)	52 ± 1(59.8)
Cyclic AMP	466 ± 7***	55 ± 1***(11.7)	296 ± 6***(63.6)	115 ± 0.5***(24.6)
Glucagon	365 ± 7.5***	44 ± 1***(12.0)	214 ± 4.5***(58.5)	108 ± 3***(29.5)
Isoproterenol	170 ± 3.5***	17 ± 0.5***(10.0)	70 ± 2.5*** (41.0)	84 ± 1.5***(49.0)
B) Exogenous ALA present				
None	710 ± 17	42 ± 1(6.0)	78 ± 5(11.0)	590 ± 17(82.5)
Cyclic AMP	700 ± 10	111 ± 1.5***(16.0)	430 ± 7***(61.5)	157 ± 5***(22.5)
Glucagon	667 ± 7.5	99 ± 2***(14.8)	330 ± 10***(49.4)	236 ± 11**(35.3)
Isoproterenol	657 ± 3.5	62 ± 2**(9.4)	160 ± 11*(24.4)	435 ± 11(66.2)

Cultures of male rat hepatocytes were prepared in multiwell plates. Additions (including ALA, 25 μg/mL) made at 24 h; 19 h later the concentration and composition of the accumulating porphyrins was determined. The total porphyrins are given as averages ± SEM of four observations obtained in one experiment. The effect of glucagon (employed here at a final concentration of 28 μg/mL) was confirmed in another experiment, using three different concentrations (5.7, 32 and 57 μg/mL, all three shown to be equally effective). c-AMP and isoproterenol were both added to a final concentration of 50 μM.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, when compared to corresponding control values

component (1 μg/mL) of the medium and the extra dose of 10 μg/mL was omitted: the effect of T_3 was then similar, but less pronounced.

Quantitative relationship between coproporphyrin and protoporphyrin concentration in the hepatocyte cultures examined in this work

Although an increase in total porphyrins was consistently noted with c-AMP in six different experiments, these increases varied between 3-fold (see, for example, Table 2) and 8.5-fold (as in Fig. 3). Likewise, the accumulation of coproporphyrin in the presence of exogenous ALA, although consistently seen, varied from a value of 23% of total porphyrins (Fig. 2) to >60 (see Table 1). However, when the percentage of coproporphyrin found in the presence of exogenous ALA was plotted against the percentage of protoporphyrin in the same culture, an inverse relationship was found between the two, with the same line of correlation encompassing all control and coproporphyric cultures, whether c-AMP, glucagon or T_3 (or combinations thereof) had been used to induce coproporphyria (Fig. 5A). The same relationship also applied when the absolute values of the two porphyrins were compared (Fig. 5B). These findings

Table 2 Effect of T_3 added on its own or together with c-AMP on the porphyrin content of rat hepatocyte cultures and on the conversion of exogenous ALA to porphyrins. Male rat hepatocyte cultures were prepared in multiwell plates as described in the "Experimental" section; at 4 h the original serum-containing medium was changed to serum-free medium to which T_3 had been added, where indicated, at a concentration of 1 μg/mL. At 22 h the serum-free medium (containing, where necessary, T_3 at the same concentration) was changed, and a further dose of T_3 (10 μg/mL) added, where appropriate, at 29 h. ALA (25 μg/mL of culture) and c-AMP (50 nmol/mL) were added at 24 h, and the porphyrins accumulating in cells and media determined at 43 h. Results are averages ± SEM of four observations obtained in one experiment which was confirmed three times

Additions	Porphyrins of combined cells and medium (pmol/mg protein)			
	Total	Uro	Copro	Proto
		(percent of total given in brackets)		
A) Exogenous ALA absent				
None	173 ± 22	18 ± 2.5(10.2)	54 ± 10(31.0)	100 ± 9(57.9)
T_3	266 ± 11	22.5 ± 1(10)	129 ± 8***(49.2)	74 ± 2.5*(32.7)
c-AMP	575 ± 6	61 ± 1(10.5)	357 ± 7.5(62.1)	157 ± 2.5(27.4)
T_3 + c-AMP	762 ± 23***	70 ± 1.5**(9.1)	615 ± 22*** (30.6)	78 ± 1.5***(10.2)
B) Exogenous ALA present				
None	775 ± 22	66 ± 1(8.5)	100 ± 5(12.8)	608 ± 21(78.5)
T_3	742 ± 7.5	78 ± 1***(10.5)	270 ± 8.5***(36.5)	393 ± 16***(52.9)
c-AMP	775 ± 12	125 ± 6(16.2)	453 ± 16(56.2)	214 ± 12(27.6)
T_3 + c-AMP	889 ± 21**	113 ± 3(12.7)	664 ± 17***(75.0)	112 ± 3.5***(12.6)

Uro Uroporphyrin; *Copro* Coproporphyrin; *Proto* Protoporphyrin

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, when compared to corresponding values obtained in the absence of T_3

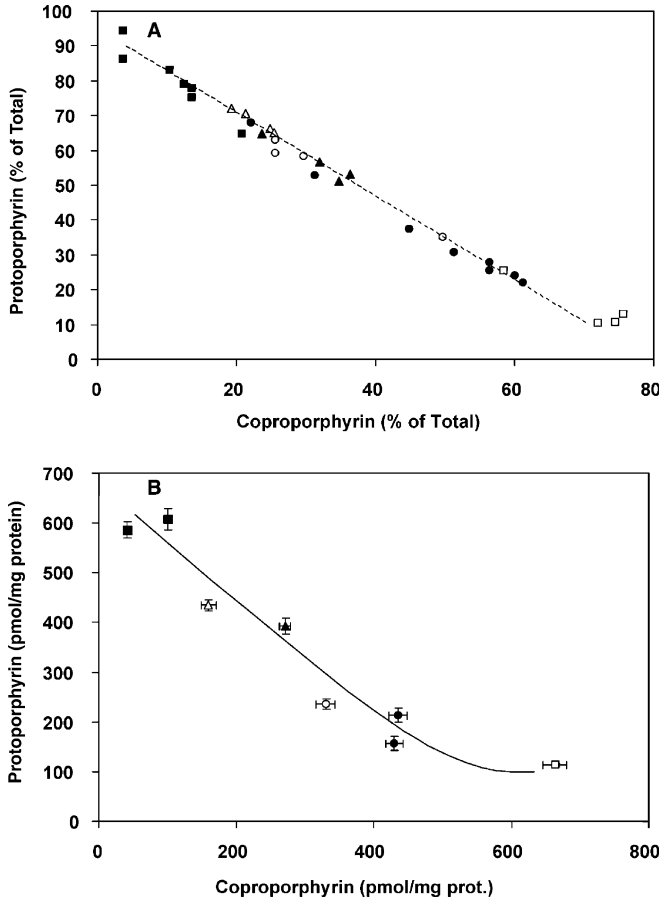


Fig. 5A–B Relationship between the amounts of protoporphyrin and coproporphyrin in rat hepatocyte cultures incubated with ALA (25 μg/mL of culture), either on its own (*filled squares*), or together with agent(s) inducing preferential accumulation of coproporphyrin: ALA + c-AMP (*filled circles*); ALA + T3 (*filled triangles*); ALA + c-AMP + T3 (*unfilled squares*); ALA + glucagon (*unfilled circles*); ALA + isoproterenol (*unfilled triangles*). The percentage of total porphyrin are given in **A**, each symbol representing an individual observation. The absolute values of the two porphyrins are given in **B** instead (as the average ± SEM values of the experiments of Tables 1,2)

support the interpretation that c-AMP and T_3 cause coproporphyrin to accumulate by inhibiting its further metabolism to protoporphyrin.

General conclusions

In this paper, we report that c-AMP and c-AMP-releasing agents induce marked accumulation of coproporphyrin in cultures of rat hepatocytes, and provide evidence that inhibition of coproporphyrinogen III metabolism is most probably involved. The activity of coproporphyrinogen oxidase was found to be unaltered, but this does not exclude the possibility (1) of a reversible inhibitor of the enzyme, which is active in the whole cell in vivo, and becomes displaced as soon as the cells are homogenized. Alternatively, (2) cyclic AMP might hinder access of coproporphyrinogen III to the oxidase by inhibiting transfer into the mitochondrion, or (not shown in Scheme 1) by accelerating extrusion of coproporphyrinogen into the extracellular compartment. Finally, (3) it may promote oxidation of coproporphyrinogen to coproporphyrin, which cannot be metabolized and would therefore be expected to accumulate (see Scheme 1). The data we have provided in this paper cannot distinguish between these various possible mechanisms, and additional work, involving measurements of mitochondrial respiration, production of reactive oxygen species and, more directly, the rate of oxidation of coproporphyrinogen, will be required (as well as—possibly—a study of the transfer of coproporphyrinogen across cellular membranes).

We currently favour mechanism (3) above, for the following reasons:

(a) A similar mechanism of "oxidative escape" of uroporphyrinogen has been proposed, at least as a contributing factor, in drug-induced uroporphyria and the corresponding human condition, *porphyria cutanea tarda* of the sporadic variety (Heikel et al 1958; Sinclair et al 1987; De Matteis et al 1988), and there is now evidence for the activation of an oxidative stress mechanism in this condition (reviewed in De Matteis 1998), with various biological molecules undergoing oxidative changes. In addition to uroporphyrinogen (Sinclair et al 1998), these include bilirubin (De Matteis et al 2002) and, under certain conditions, DNA bases (Faux et al 1992). The finding (Lambrecht et al 1987) that uroporphyrin accumulates in chicken hepatocytes made uroporphyric by drugs, but that little or no inhibition of uroporphyrinogen decarboxylase can be detected, offers a clear parallel with the lack of inhibition of coproporphyrinogen oxidase now found in rat hepatocytes treated with c-AMP.

(b) Isaacs and Binkley (1977) have reported increased production of peroxides in the livers of rats subjected to starvation or treated with c-AMP in vivo. There is evidence that fasting (a condition known to increase glucagon and liver c-AMP levels) is accompanied by lower hepatic content of GSH, a cellular protective molecule present both in the cytosol and in the mitochondria (Reed 1995). In addition, both fasting and administration of c-AMP will increase the liver activity of haem oxygenase (Bakken et al 1971), a heat shock protein known to be induced under conditions of oxidative stress (Maines 1992).

(c) Finally, this general mechanism gains some indirect support from the reported effects of T_3, since this hormone, as discussed above, is known to stimulate oxygen consumption and to increase the production of oxygen radicals within the mitochondria. Although coproporphyrin clearly predominated after treatment with c-AMP, accumulation of uroporphyrin and, in some cases, of protoporphyrin was also seen to some extent. This suggests that any

reoxidation mechanism may extend to other pools of porphyrins which exist as intermediary porphyrinogens. Judging from the intracellular distribution of the enzymes of haem biosymthesis (Scheme 1), uroporphyrinogen is assembled and decarboxylated in the cytosol, whereas coproporphyrinogen (and protoporphyrinogen) are metabolized within the mitochondrion. Perhaps the main site of production of oxidative species, whether cytosolic/microsomal or mitochondrial, will determine which porphyrinogen (uroporphyrinogen or coproporphyrinogen) would be most likely to suffer oxidation and accumulate. The effects of c-AMP now described may be relevant to the stimulation of porphyrin biosynthesis caused by fasting in genetic acute porphyria and in the corresponding experimental models induced by drugs. A partial block in coproporphyrinogen metabolism would further decrease the concentration of haem in the liver, resulting in greater stimulation of hepatic ALA-synthase and contributing to the risk of an acute attack.

It should be noted that the concentrations of c-AMP shown to be active on coproporphyrin accumulation in this present work (1–50 μM, EC_{50}∼3 μM) are within the range of dibutyryl c-AMP concentrations found to be effective, in cultured cells, in inhibiting thymidine incorporation into DNA or cholesterol ester hydrolase (Michnoff et al 1983; Ruiz et al 1990). They also resemble the intracellular c-AMP concentrations achieved by stimulants in vivo (Albert et al 1989).

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References

Albert B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1989) Molecular Biology of the cell, 2nd edn. Garland, New York, pp 695

Bakken AF, Thaler MM, Pimstone NR, Schmid R (1971) Stimulation of hepatic heme oxygenase by fasting and by hormones. Gastroenterology 60:177

Barbe P, Larrouy D, Boulanger C, Chevillotte E, Viguerie N, Thalamas C, Oliva Trastoy M, Roques M, Vidal H, Langin D (2001) Triiodothyronine-mediated up-regulation of UCP2 and UCP3 mRNA expression in human skeletal muscle without coordinated induction of mitochondrial respiratory chain genes. FASEB J 15:13–15

Castilho RF, Kawaltowski AJ, Vercesi AE (1998) 3,5,3'-triiodothyronine induces mitochondrial permeability transition mediated by reactive oxygen species and membrane thiol oxidation. Arch Biochem Biophys 354:151–157

De Matteis F (1998) Porphyria cutanea tarda of the toxic and sporadic varieties. Clin Dermat 16:265–275

De Matteis F, Harvey CC (1987) Cyclic AMP-dependent accumulation of coproporphyrin III in cultures of rat hepatocytes. Bollettino Istituto Dermatologico S. Gallicano 13:337–338

De Matteis F, Zetterlund P, Wetterberg L (1981) Brain 5-aminolevulinate synthase. Biochem J 196:811–817

De Matteis F, Manno M, Harvey C, Ferioli A (1987) A high performance liquid chromatography technique for separation of porphyrins from porphyric hepatocytes. In: Foa' V, Emmett EA, Maroni M, Colombi A (eds) Occupational and environmental chemical hazards. Ellis Horwood, Chichester, pp. 284–286

De Matteis F, Harvey C, Reed C, Hempenius R (1988) Increased oxidation of uroporphyrinogen by an inducible liver microsomal system. Biochem J 250:161–169

De Matteis F, Dawson SJ, Pons N, Pipino S (2002) Bilirubin and uroporphyrinogen oxidation by induced cytochrome P4501 A and cytochrome P4502B. Role of polyhalogenated biphenyls of different configuration. Biochem Pharmacol 63:615–624

Edwards AM, Elliott WH (1974) Induction of δ-aminolevulinic acid synthetase in isolated rat liver cell suspensions. Adenosine 3′:5′-monophosphate dependence of induction by drugs. J Biol Chem 249:851–855

Faux SP, Francis JE, Smith AG (1992) Induction of 8-hydroxydeoxyguanosine in Ah-responsive mouse liver by iron and Aroclor 1254. Carcinogenesis 13:247–250

Ferioli A, Harvey C, De Matteis F (1984) Drug-induced accumulation of uroporphyrin in chicken hepatocyte cultures. Biochem J 224:769–777

Fernandez V, Videla LA (1993) Influence of hyperthyroidism on superoxide radical and hydrogen peroxide production by rat submitochondrial particles. Free Radic Res Comm 18:329–335

Grandchamp B, Nordmann Y (1982) Coproporphyrinogen III oxidase assay. Enzyme 28:196–205

Grandchamp B, Deybach JC, Grelier M, De Verneuil H, Nordmann Y (1980) Studies of porphyrin synthesis in fibroblasts of patients with congenital erythropoietic porphyria and one patient with homozygous coproporphyria. Biochem Biophys Acta 629:577–586

Heikel T, Lockwood W, Rimington C (1958) Formation of non-enzymic haem. Nature (London) 182:313

Isaacs JT, Binkley F (1977) Cyclic AMP-dependent control of the rat hepatic glutathione disulfide-sulfhydryl ratio. Biochim Biophys Acta 498:29–38

Lambrecht R, Sinclair P, Bement WJ, Sinclair J, Carpenter H (1987) Comparison of uroporphyrinogen decarboxylase activities and uroporphyrin accumulation in mice, Japanese quail and cultured chick embryo hepatocytes treated with polyhalogenated aromatic compounds. Ann NY Acad Sci 514:337–338

Lim CK, Peters TJ (1988) High-performance liquid chromatography of porphyrins. J Chromatogr 429:123–152

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Maines MD (1992) Heme oxygenase. CRC, Boca Raton, FL, pp 156–159

Marks GS (1986) Exposure to toxic agents:the heme biosynthetic pathway as indicator. Crit Rev Toxic 15:151–170

Michnoff CA, de la Houssaye BA, Masaracchia RA (1983) Kinetics of adenosine 3′, 5′-monophosphate-dependent protein kinase activation and inhibition of thymidine incorporation into DNA in P1798 lymphosarcoma cells. Cancer Res 43:3514–3520

Paine AJ, Legg RF (1978) Apparent lack of correlation between loss of cytochrome P-450 in hepatic parenchymal cell culture and the stimulation of haem oxygenase activity. Biochem Biophys Res Comm 81:672–679

Papa S (2002) The NDUFS4 nuclear gene of complex I of mitochondria and the c-AMP cascade. Biochim Biophys Acta 1555:147–153

Papa S, Scacco S, Sardanelli AM, Petruzzella V, Vergari R, Signorile A, Technokova-Dobrova Z (2002) Complex I and the c-AMP cascade in human physiopathology. Biosci Rep 22:3–16

Rabelo R, Schifman A, Rubio A, Sheng X, Silva JE (1995) Delineation of thyroid hormone-sensitive sequence within a critical enhancer in the rat uncoupling protein gene. Endocrinology 136:1003–1013

Raha S, Myint AT, Johnstone L, Robinson BH (2002) Control of oxygen free radical formation from mitochondrial complex I:role for protein kinase A and pyruvate dehydrogenase kinase. Free Radic Biol Med 32:421–430

Reed DJ (1995) Toxicity of oxygen. In: De Matteis F, Smith LL (eds) Molecular and cellurar mechanisms of toxicity. CRC, Boca Raton, FL, pp 35–68

Ruiz MB, Ochoa B, Lacort M (1990) Glucagon- and dibutyryl cyclic AMP-produced inhibition of cholesterol ester hydrolase in isolated rat hepatocytes: role of calcium. J Biochem 107:476–479

Sano S, Granick S (1961) Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J Biol Chem 236:1173–1180

Sinclair PR, Lambrecht R, Sinclair J (1987) Evidence for cyrochrome P450-mediated oxidation of uroporphyrinogen by cell-free extracts from chick embryos treated with 3-methylcholanthrene. Biochem Biophys Res Comm 146:1324–1329

Sinclair PR, Gorman N, Dalton T, Walton HS, Bement WJ, Sinclair JF Smith AG, Nebert DW (1998) Uroporphyria produced in mice by iron and 5-aminolevulinic acid does not occur in Cyp1a2 (-/-) null mutant mice. Biochem J 330:149-153

Smith AG, De Matteis F (1990) Oxidative injury mediated by the hepatic cytochrome P450 system in conjunction with cellular iron. Effects on the pathway of haem biosynthesis. Xenobiotica 20:865–877

Smith AG, Francis JE (1993) Gentic variations of iron-induced uroporphyria in mice. Biochem J 291:29–35

Tsuboi T, da Silva Xavier G, Holz GG, Jouaville LS, Thomas AP, Rutter GA (2003) Glucagon-like peptide-1 mobilizes intracellular Ca2+ and stimulates mitochondrial ATP synthesis in pancreatic MIN6 beta-cells. Biochem J 369:287–299

Venditti P, Daniele MC, Masullo P, Di Meo S (1999) Antioxidant-sensitive triiodothyronine effects on characteristics of rat liver mitochondrial population. Cell Physiol Biochem 9:38–52