Uroporphyria development in cultured chick embryo fibroblasts long-term treated with chloramphenicol and ethidium bromide

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Long-term chloramphenicol- and ethidium bromide-treated chick embryo fibroblasts synthesize large amounts of porphyrins from exogenously added σ-aminolevulinic acid. The porphyrins consist mainly of uro- and heptacarboxyporphyrins and are retained within cells. Uroporphyria development is a time-dependent process which accompanies a step-wise decrease in the capacity of the mitochondrial respiratory chain. Upon removal of chloramphenicol from the medium, the pattern of porphyrin production readily returns to normal (mainly proto- and coproporphyrins found in the medium) while ethidium bromide-treated cells remain uroporphyric. The results suggest that impairment of mitochondrial functions in chicken by xenobiotics leads to uroporphyria development.

Porphyria *Uroporphyrin* *Mitochondria* *Respiration* *Chicken*

1. INTRODUCTION

A variety of chemical compounds have been shown to induce porphyria in mammals and birds (review [1]). Among the xenobiotics tested, polyhalogenated aromatic hydrocarbons (PHA) are known to cause a massive production of uro- and heptacarboxyporphyrins in the liver of treated animals (review [2]). This type of porphyria is a time-dependent process which usually takes at least a week in birds [3]. It resembles a syndrome known in man as porphyria cutanea tarda, characterized by a massive accumulation of uroporphyrin in the liver and a marked uroporphyrinuria [4,5]. Experimental uroporphyria can also be induced by PHA in chick embryo liver cells in culture [1]. When the cells are incubated in the presence of σ-aminolevulinate (ALA), the development of uroporphyria is a function of the concentration of xenobiotics and appears over a period of less than 24 h [6,7]. The mechanism whereby PHA induce uroporphyria in birds as well as mammals in vivo and in vitro is still obscure, but the data obtained thus far show that this phenomenon is specifically related to the reduction in activity of uroporphyrinogen decarboxylase [2].

We have reported [8–10] that cultured chick embryo fibroblasts (CEP) are inherently resistant to the growth-inhibitory effect of chloramphenicol (CAP) and ethidium bromide (EtdBr), inhibitors of protein synthesis on mitoribosomes and mitochondrial DNA (mtDNA) replication and transcription, respectively. Long-term drug-treated cells are respiration-deficient and devoid of mitochondrial cytochromes *b* and aa_3. The protein moieties of these cytochromes are known to be coded for either completely (*b*) or in part (aa_3) by mtDNA [11]. In the course of a study on the effect of CAP and EtdBr on the mitochondrial cytochrome content of CEF, the syntheses of the heme precursor moieties of these proteins have been investigated. We here demonstrate that CAP and EtdBr induce uroporphyria in CEF incubated in the presence of exogenously-added ALA. It is a time-dependent process which is fully reversible when CAP but not EtdBr is eliminated from the culture medium.

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2. MATERIALS AND METHODS

Growth medium, sera and antibiotics were purchased from Gibco. CAP, EtdBr and ALA were obtained from Sigma. Porphyrin standards were from Porphyrin Products. Methanol and water (HPLC grade) were obtained from Fisher.

Chick embryo fibroblasts were prepared as in [8] and propagated in Ham's F_{12} medium supplemented with antibiotics, sera and uridine as in [12] and, where indicated, CAP (100 μg/ml) or EtdBr (0.4 μg/ml). Cells in mid to late log phase were used to study the biosynthesis of porphyrins from ALA (1.2 mM). They were incubated in fresh growth medium without phenol red and harvested 24 h later by trypsinization. The cells ($2-6 \times 10^7$) were washed twice with PBS and resuspended in 2 ml distilled water. The cell suspension was adjusted to pH 3.2 with saturated sodium acetate and the porphyrins extracted 3 times with 2 ml ethyl acetate–glacial acetic acid (4:1, v/v). The extracts were evaporated under nitrogen, the residues taken up in 500 μl of 1 N HCl and centrifuged for 40 min at 15000 rpm. The porphyrins in 10 ml growth medium were similarly extracted 3 times with 5 ml ethyl acetate–glacial acetic acid at pH 3.2. Control experiments with various amounts of a mixture of porphyrins with 8–2 carboxyl groups revealed that the percent recovery of the extracted porphyrins varied from 80% (uroporphyrin) to 98% (protoporphyrin). Porphyrin composition was determined by high-pressure liquid chromatography (HPLC) using a Lichrosorb RP-18 column (5 μ) essentially as in [13]. The experiments were performed with Model 334 Beckman Liquid Chromatograph. The eluting materials were detected by absorption at 404 nm using a Hitachi Spectrophotometer Model 155, recorded and the surface area computerized with a Perkin-Elmer Sigma 10. Uroporphyrin isomer analysis was performed as in [14] but modified by Dr A. Sasarman (personal communication). Cytochrome spectra were performed as in [8]. The protein concentration was determined according to [15].

Table 1

Porphyrin formation from exogenous ALA by control and long-term CAP- and EtdBr-treated chick embryo fibroblasts

Drug	Distribution and profile of porphyrins (pmol/mg cell protein)									
	Cell	Medium	Total		C_8	C_7	C_6	C_5	C_4	C_2
None (8)	102 ± 60	915 ± 203	1017 ± 183	I	61	29	0	0	0	12
				E	4	45	52	7	197	610
CAP$^+$ (8)	831 ± 151	612 ± 194	1443 ± 282	I	630	199	0	0	0	2
				E	49	182	88	23	83	187
EtdBr$^+$ (5)	870 ± 477	424 ± 200	1294 ± 574	I	626	237	4	0	0	3
				E	16	78	4	0	73	253
EtdBr$^-$ (4)	579 ± 335	580 ± 123	1159 ± 387	I	432	138	2	0	0	7
				E	36	169	82	38	72	183
CAP$^-$ (4) (24 h)	384 ± 113	672 ± 260	1056 ± 319	I	279	97	1	0	0	7
				E	23	96	29	4	154	366
CAP$^-$ (2) (48 h)	84 ± 25	565 ± 103	649 ± 79	I	38	21	1	1	1	22
				E	0	0	64	0	210	291

Total porphyrin content of cells and medium and their profile were determined by HPLC [13]. Values represent the mean ± SD of individual experiments. The number of experiments are given in parentheses. $^+$, cells maintained in the presence of the drugs for more than 21 days; , cells shifted to drug-free medium for 24 or 48 h and 21 days (EtdBr); I, intracellular; E, extracellular; C_8–C_2, porphyrins with 8–2 carboxyl groups

3. RESULTS

When cultivated in the presence of ALA, control CEF synthesized large amounts of porphyrins (table 1). They consisted mainly of proto- (61%) and coproporphyrins (19%) and were almost exclusively located in the medium. Small amounts of porphyrins with 8–2 carboxyl groups were also detected. Uro- and heptacarboxyporphyrins were the major heme precursor present in the cells. These observations bear a close resemblance to those reported recently concerning cultured chick embryo liver cells incubated in the presence of ALA [6,7].

The amount, distribution and profile of porphyrins were found to be substantially affected in long-term CAP- and EtdBr-treated CEF. As shown in table 1, drug-treated cells synthesized on average 20–40% more porphyrins than control cells. Further, more than 60% of the total amount of porphyrins produced were retained within cells. The latter appeared to contain predominantly porphyrins, rather than porphyrinogens, since the color of the freshly trypsinized cells was reddish. The type III isomers of uro- (50%) and heptacarboxyporphyrins (25%) constituted the major heme precursors synthesized and were found primarily in the cells. Accumulation of proto- and coproporphyrins was greatly reduced compared to control cells and these compounds were found exclusively in the medium. Variable amounts of porphyrins with 8–2 carboxyl groups were also present in the medium.

The development of uroporphyria in CEF was found to be a slow process (fig.1) which began at about day 10, after 6–7 cell generations, after the mitochondrial respiratory capacity of the cells had been severely perturbed [16]. Uroporphyria was clearly established at day 20, 6–7 generations later. The inhibitory effect of CAP and EtdBr on the synthesis of proteins coded for by mtDNA resulted in the formation of mitochondria devoid of a functional respiratory chain. This is illustrated by comparing the reduced-minus-oxidized spectra of total cytochromes of control cells to those of the drug-treated cells. As shown in fig.2, the characteristic bands of cytochromes aa_3 (at 603 nm) and *b* (shoulder at 562 nm) (trace A) disappeared in CAP- and EtdBr-treated cells (traces B and E, respectively) while that of cytochrome *c* (at 548 nm), a nuclearly coded protein, is still present.

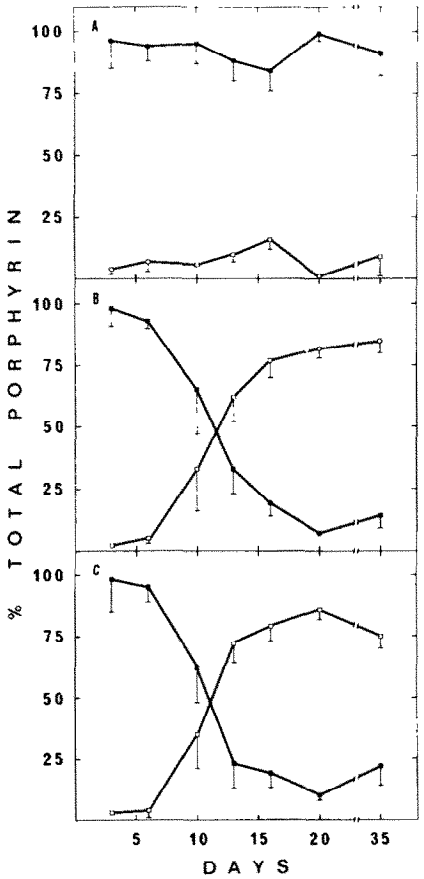

Fig.1. Time-dependent porphyrin formation from exogenous ALA by control (A) and long-term CAP (B)- and EtdBr (C)-treated CEF. Experimental conditions were as in section 2. Values represent the mean ± SD of 3 individual experiments, except for day 35 which represent the mean ± SD of 8 (A and B) and 4 (C) different experiments performed between days 30 and 40. (■—■) Uro- and heptacarboxyporphyrins, (□—□) copro- and protoporphyrins.

The distribution as well as the profile of the porphyrins were found to return to normal when CAP was eliminated from the culture medium (table 1). These time-dependent processes were completed at 48 h and accompanied the restoration of the synthesis of mtDNA-coded proteins as illustrated by the reappearance of the absorption bands of mitochondrial cytochromes aa_3 and *b* (fig.2, traces C,D). In contrast, when EtdBr was removed from the culture medium, protein synthesis on mitoribosomes was not restored (fig.2, trace F)

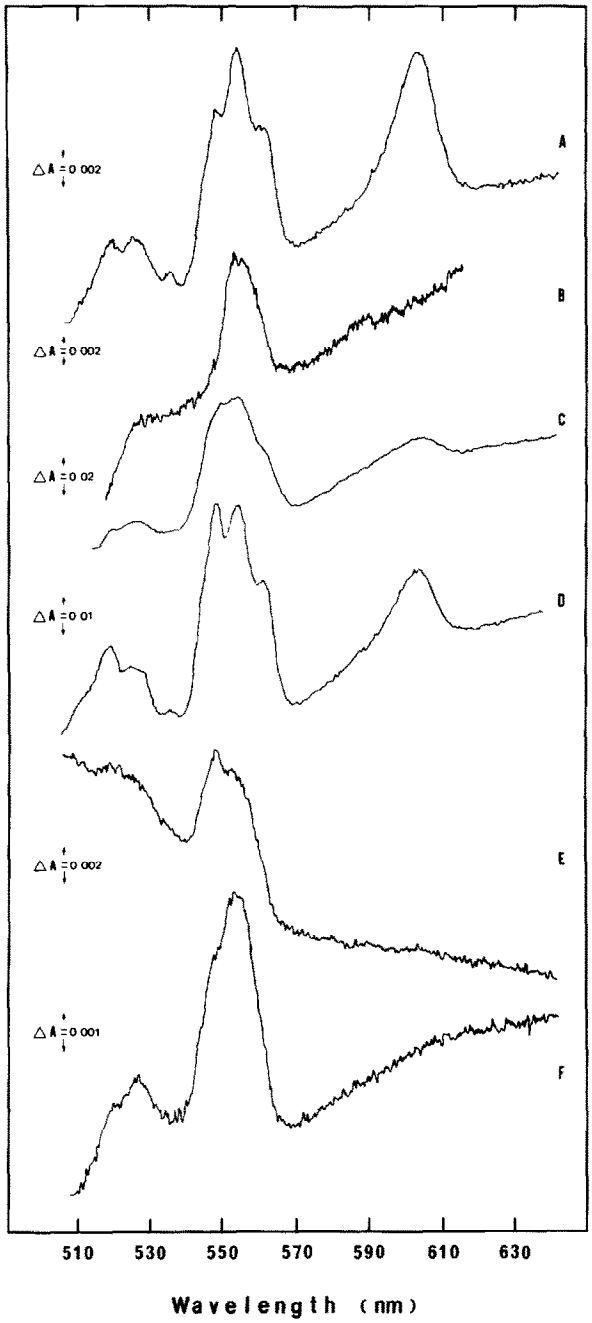

Fig.2. The reduced-minus-oxidized spectra at liquid nitrogen temperature of the cytochromes of control (A) and long-term CAP (B)- and EtdBr (F)-treated CEF. (C and D) CAM-treated cells shifted to drug-free medium for 24 and 48 h, respectively. (F) EtdBr-treated cells shifted to drug-free medium for 23 days. For traces A–F, the concentrations of cells ($\times 10^7$) per ml were 15.9, 10.8, 9.9, 13.2, 9.6 and 10.5, respectively.

and the cells continued to accumulate uro- and heptacarboxyporphyrins (table 1): EtdBr treatment of CEF results in the irreversible loss, to less than one copy per cell, of mtDNA molecules (in preparation).

4. DISCUSSION

This study demonstrates that long-term treatment of CEF with CAP and EtdBr results in disturbance of the decarboxylation of uroporphyrinogen and in the accumulation of uro- and heptacarboxyporphyrins in the treated cells. It is a time-dependent process which becomes clearly evident 2–3 weeks after the beginning of the treatment. The step-wise decrease in the capacity of CEF to decarboxylate uroporphyrinogen appears to accompany the step-wise decrease in the capacity of the mitochondrial respiratory chain. When CAP is eliminated from the culture medium, the pattern of porphyrin production readily returns to normal as do protein synthesis on mitoribosomes and electron transport along the mitochondrial respiratory chain. In contrast, EtdBr-treated cells shifted to EtdBr-free medium remain uroporphyric and respiration deficient. These results suggest the possibility that impairment of respiration and/or other mitochondrial functions by xenobiotics can lead to uroporphyria development in tissues of those birds sensitive to the action of these compounds.

That mitochondria can be involved in the development of uroporphyria is a possibility that has been evoked by other investigators [2,3,17]. However, no direct correlation has yet been established between the impairment of mitochondrial functions and the generally accepted idea that uroporphyria induced in mammals and birds by xenobiotics is caused by decreased activity of uroporphyrinogen decarboxylase. Speculations on the mechanism of action of these compounds include decreased synthesis and/or increased catabolism of uroporphyrinogen decarboxylase ([18], but see [19]), direct inhibition of the enzyme by binding of the xenobiotics and/or their metabolites to a group essential for catalytic activity [20], inactivation of the enzyme by formation of oxygen radicals [21] and alterations of the redox state of the cells leading to decreased ability to maintain uroporphyrinogen and/or the catalytic

thiol group of the enzyme in the reduced form (review [2]). Considering the fact that CEF can be cultivated for long periods in the presence of CAP and EtdBr and that the capacity of the cells to decarboxylate uroporphyrinogen can be modulated by CAP, CEF appear to provide a good model system to obtain some insight into these problems. Work is in progress to further our understanding of the development of uroporphyria in CEF.

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