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SHORT COMMUNICATIONS

Enhancement of 3-methylcholanthrene-mediated induction of cytochrome P-450 by 17α-ethynylestradiol: immunochemical studies

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Cytochromes P-450 are a family of monooxygenases involved in the oxidative metabolism of many endogenous and exogenous substrates. Induction of cytochrome P-450 plays an important role in either detoxification or activation of many foreign substances. A number of isozymes can be induced in the livers of animals by treatment with xenobiotics (for reviews, see Refs 1–3). We have reported previously that, in chick embryo hepatocytes in culture, induction of cytochrome P-450 by 3-methylcholanthrene or β-naphthoflavone is potentiated by 17α-ethynylestradiol (EE_2) [4]. Enzymatic studies suggested that the form of cytochrome P-450 induced by the combination of EE_2 and either 3-methylcholanthrene (MC) or β-naphthoflavone was the same form as that increased by MC alone. Recently we purified a protein of 57,000 mol. wt from chicken embryos treated with MC (designated P-450_{MC57K}) [5]. Rabbit antibodies prepared against this protein completely inhibit ethoxyresorufin-*O*-deethylase (EROD) activity in hepatic microsomes from MC-treated chicken embryos [5]. In this study, we used this antibody to compare the antigenic homologies of the forms of cytochrome P-450 induced in cultured chick hepatocytes by MC and EE_2 versus MC alone. Antibody inhibition of EROD activity suggested that the combination of MC and EE_2 induced the same form of cytochrome P-450 as that induced by MC alone.

Materials and methods

Primary cultures of livers from 16- to 17-day-old chicken embryos were prepared and treated with MC (0.93 μM) and EE_2 (15 μM) as described previously [4]. Microsomes were prepared from cultured cells, and cytochrome P-450 and EROD were measured in the microsomes as described previously [4]. Protein was determined by the method of Lowry *et al.* [6], using bovine serum albumin as a standard. Microsomal proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 5–10% gradient gel, and immunoblots were prepared as described [5]. Immunoinhibition of EROD activity was determined by the method of Lubet *et al.* [7]. Briefly, liver microsomes from cultured chick embryo hepatocytes (25 μg protein/reaction) were incubated with increasing amounts of preimmune serum or serum from rabbits immunized with either purified cytochrome P-450_{MC57K}, or with a 50K cytochrome P-450 purified from glutethimide-treated embryos (P-$450_{GLUT50K}$). Preimmune serum was present in each mixture to maintain a constant amount of serum. After 20 min of incubation at room temperature, EROD activity was measured as described previously [4].

Results and discussion

Two microsomal proteins of 57K and 55K mol. wt were induced by MC (Fig. 1, lane b). These two proteins were not detected in microsomes from cells that were either untreated or exposed to EE_2 alone (Fig. 1, lanes c and d). However, combined treatment of cells with EE_2 and MC caused further increases in the proteins of 57K and 55K compared to treatment with MC alone (Fig. 1, lane a vs lane b), with a greater increase in the protein of 55K mol. wt.

Antiserum prepared towards chick cytochrome P-450_{MC57K} detected a microsomal protein of 57K mol. wt on immunoblots (Fig. 2) and completely inhibited MC-induced EROD activity in microsomes prepared from cultured chick hepatocytes (Fig. 3), indicating that the forms of cytochrome P-450 induced by MC in chick hepatocytes *in ovo* and in cultures are identical. The additional proteins recognized by the antiserum were probably not cytochromes P-450 due to their molecular weight and the lack of any increase after treatment of the cells with MC.

The antiserum detected a greater increase in the 57K protein following the treatment of cells with both EE_2 and MC than with MC alone (Fig. 2, Table 1) and completely inhibited microsomal EROD activity induced by the combination of MC and EE_2 (Fig. 3). At equal ratios of serum per mole of cytochrome P-450 the extent of inhibition was identical (Fig. 3), providing evidence that the cytochrome P-450 induced by MC and EE_2 versus MC alone share identical epitopes required for catalytic activity. No inhibition of microsomal EROD activity was observed in microsomal preparations incubated with preimmune serum prepared from the same rabbit prior to immunization or with immune serum prepared towards cytochrome P-$450_{GLUT50K}$ (results not shown).

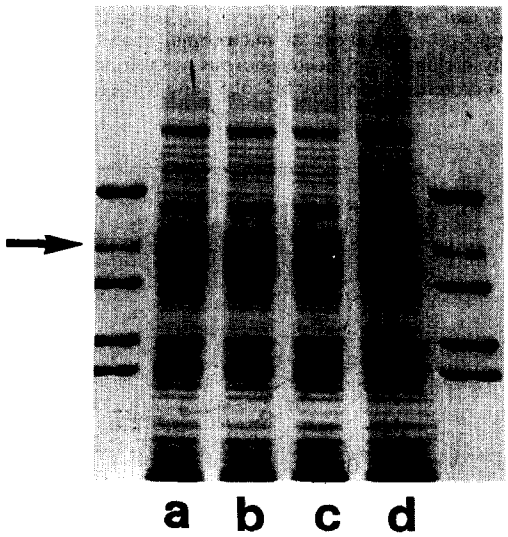

Fig. 1. Electrophoretic analysis of microsomes from cultured hepatocytes by SDS–PAGE. Microsomes were prepared from cells treated with EE_2 (15 μM); MC (0.93 μM); or MC and EE_2 for 24 hr. Microsomal protein (15 μg) was applied to each well. Lane a, MC + EE_2; lane b, MC; lane c, EE_2; lane d, control. Molecular weight markers: BSA, 66,000; catalase, 57,000; fumarase, 49,000; aldolase, 41,000; and lactate dehydrogenase, 36,000. The arrow indicates the position of proteins of 57,000 molecular weight.

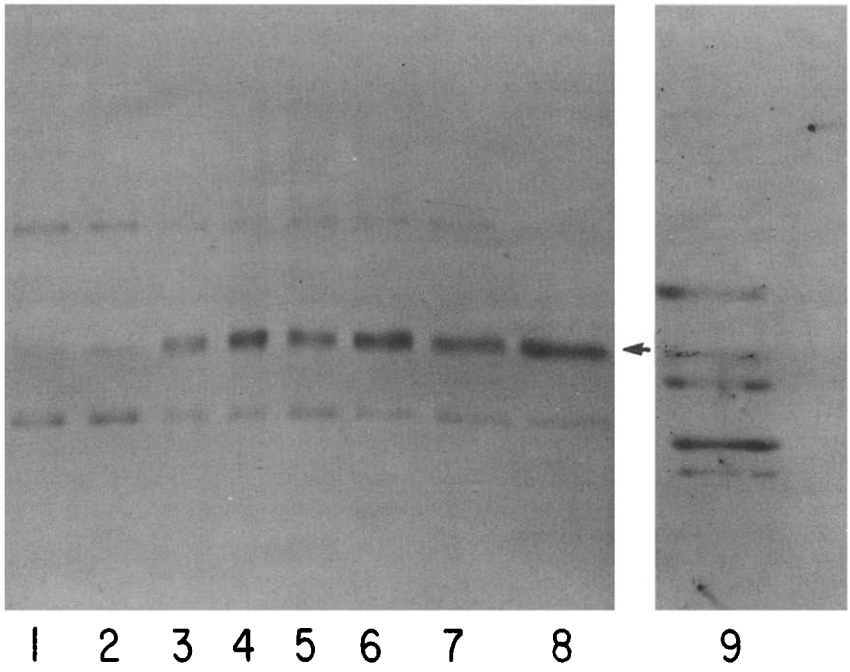

Fig. 2. Immunoblot of microsomes from cultured hepatocytes. Microsomes were from the same experiment as in Fig. 1 and were treated with antiserum to P-450$_{MC57K}$, as described in Materials and Methods. Lane 1, control (15 μg protein); lane 2, EE_2 (15 μg protein); lane 3, MC (5 μg protein); lane 4, MC + EE_2 (5 μg protein); lane 5, MC (10 μg protein); lane 6, MC + EE_2 (10 μg protein); lane 7, MC (15 μg protein); lane 8, MC + EE_2 (15 μg protein); and lane 9, molecular weight markers, stained with India ink. The arrow indicates the position of proteins of 57,000 molecular weight.

Previously, we had reported that MC induces only one microsomal protein in cultured chick hepatocytes in the 55K mol. wt region [9]. However, by using a 5–10% separating gel in analysis of microsomal proteins by SDS–polyacrylamide gel electrophoresis, we were able to detect two proteins induced by MC of 57 and 55K (Fig. 1). These two proteins of 55 and 57K may correspond to cytochromes P-450 from the IA_1 and IA_2 subfamily [2, 10, 11]. The immunoblot resolved a diffuse protein band in the region of 57K mol. wt which may also contain the 55K protein. Therefore, we cannot conclude whether the 55K mol. wt protein is a separate cytochrome P-450. The complete inhibition of EROD activity by antiserum to chick cytochrome P-450$_{MC57K}$ (Fig. 3) indicated that the antiserum recognized all the forms of cytochrome P-450 catalyzing this activity but does not definitively show whether more than one form of cytochrome P-450 is involved. In rats, unadsorbed antibody prepared against MC-induced hepatic cytochrome P-450 recognizes both cytochrome P-450IA_1 and cytochrome P-450IA_2, and completely inhibits EROD activity catalyzed by the purified forms [10, 11].

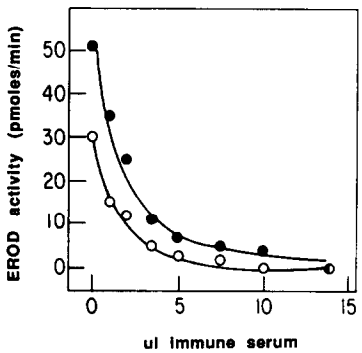

Fig. 3. Antibody inhibition of EROD activity. Microsomes (25 μg protein/reaction) from cells treated with MC (0.93 μM) (○) or MC + EE_2 (15 μM) (●) for 24 hr were incubated with preimmune and immune sera, and EROD was determined as described in Materials and Methods. Cytochrome P-450 concentrations (pmol/mg protein) were as follows: MC, 228; MC + EE_2, 372. Each symbol represents the mean of triplicate measurements, with the SD falling within the symbol. EROD activity in the absence of serum: MC, 25 ± 0; MC + EE_2, 47 ± 3.

Table 1. Quantitation of 57K mol. wt protein in immunoblots

Treatment	Relative area (arbitrary units) Microsomal protein per well (μg): 5	10	15
None	ND	ND	15
EE_2	ND	ND	23
MC	58	73	79
MC + EE_2	113	120	130

The photos in Fig. 2 were scanned with a television camera and areas were determined as described previously [8]. ND = not done.

In summary, using immunochemical techniques, we confirmed our previous report that the induction of cytochrome P-450 by MC in cultured chick hepatocytes is potentiated by EE_2. Immunological characterization suggests that the form(s) of cytochrome P-450 increased by the combined treatment with EE_2 and MC are identical to those induced by MC alone. The results may indicate the possible mechanism by which oral contraceptives are a risk factor in carcinogenesis and porphyria cutanea tarda, two disorders in which P-450 from the I family may have a role, as discussed previously [4].

Note added in proof: Hokama *et al.* (*J Biochem* **104**: 355–361, 1988) have recently found that 3,4,5,3′,4′-Pentachlorobiphenyl induces two forms of P-450 of 56K and 54K molecular weight, in chickens, which they conclude are the chicken equivalents of P-450IA, and P-$450IA_2$.

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Selective cytotoxicity of a phenolic melanin precursor, 4-*S*-cysteaminylphenol, on *in vitro* melanoma cells

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Our recent approach of using melanin precursors to design a rational chemotherapeutic agent against malignant melanoma may be of particular interest [1–6]. One would expect that, through the melanin synthesis pathway, compounds such as melanin precursors would selectively be incorporated into melanoma cells and would become toxic through conversion by tyrosinase, an enzyme unique to melanoma cells. A similar attempt to use a melanin precursor as an anti-tumor agent has been reported by a number of investigators [7–11]. They, however, utilized catecholic compounds.

Recently, we synthesized a sulfur homologue of phenol (tyrosine), cysteinylphenol (CP*), and its amine derivative, cysteaminylphenol (CAP), and tested for *in vivo* melanocytotoxicity and antimelanoma effects. We found that (a) 4-*S*-CP and 4-*S*-CAP are good substrates of mammalian tyrosinase to form melanin-like pigments, whereas their 2-*S*-isomers are not tyrosinase substrates [2, 3], (b) 4-*S*-CP and 4-*S*-CAP, in particular 4-*S*-CAP, possess *in vivo*

* Abbreviations: CP: cysteinylphenol; CAP: cysteaminylphenol; IMDM: Iscove's Modified Dulbecco's Medium; and DOPA: dihydroxyphenylalanine.