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THE INDUCTION OF CYTOCHROME P-450 AND MONOOXYGENASE ACTIVITIES IN THE CHICK EMBRYO BY 2-ACETYLAMINOFLUORENE

Nigel J. Darby, Frank R. Burnet and Alberto Lodola

Biological Laboratory, University of Kent, Canterbury, England

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Administration of 2-acetylaminofluorene to chick embryos increases the cytochrome P-450 level 3.4 fold but causes no increase in total epoxide hydrase activity or other microsomal electron transport enzymes. The induction response shows some similarity to that elicited by phenabarbitone both in terms of the monooxygenase activities induced and their inhibition characteristics. Induction of a specific cytochrome P-450 subform by this agent may increase its detoxification and in part account for the resistance of avian species to its hepatocarcinogenic effect.

2AAF is a potent hepatocarcinogen in rats producing a variety of enzymatic and ultrastructural changes (1-3). Administration of 2AAF (50mg/kg bodyweight) once daily for five days elicits a 760% increase in epoxide hydrase activity and a 40% increase in total cytochrome P-450 by the time of sacrifice on the sixth day (1). Similar dosage regimes result in disorganisation of the endoplasmic reticulum with proliferation of smooth endoplasmic reticulum (2). Gross morphological changes such as the formation of pre-neoplastic nodules are apparent after several weeks administration of 2AAF in the diet (3).

Carcinogenicity requires the activation of 2AAF by a 3-MC inducible cytochrome P-450 to produce the N-hydroxylated derivative (4-6) which is further metabolised to produce the active species (7). The guinea pig is resistant to 2AAF but not N-OH 2AAF induced hepatocarcinogenesis which suggests it lacks the activating P-450 subform (8). This is supported by

The abbreviations used are: 2-AAF, 2-acetylaminofluorene : PB, phenabarbitone : 3-MC, 3-methylcholanthrene : N-OH. 2AAF, N-hydroxy2-acetylaminofluorene : AHH, aromatic hydrocarbon hydroxylase., DMSO, dimethylsulphoxide.

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the low N-hydroxylation activity of guinea pig 3-MC inducible P-450 subforms (9).

Avian species also appear resistant to 2AAF induced hepatocarcinogenesis (10); paradoxically chick liver microsomes show both a β-napthoflavone inducible N-hydroxylation activity and a capacity for 2AAF activation in *in vitro* mutagenesis assays (11). This report details the characterisation of the acute effects of 2AAF upon the xenobiotic metabolising enzymes of the chick embryo in comparison with PB and 3-MC. We show 2AAF is an inducer of cytochrome P-450 and associated monooxygenase functions in the chick embryo. This effect may explain in part the resistance of avian species to 2AAF induced hepatocarcinogenesis.

MATERIALS AND METHODS

Treatment of Animals

Fertile egss (Ross 1, W.C. Blacklocks Ltd., Lydd, Kent) were incubated in a Western egg incubator; after 17 days PB (10mg), 2AAF (8mg) or 3-MC (2mg) were injected into the fluid surrounding the embryo in 200µl DMSO. After a further 24 hrs incubation embryos were killed by decapitation, the livers removed to ice-cold physiological saline and the sex of each animal determined (12).

Subcellular Fractionation

20% liver homogenates were prepared in 0.25M sucrose from 2g of tissue (derived entirely from male or female embryos) using a Polytron (Kinematica) at setting number 4 (3x5s bursts). Microsomes were recovered by differential centrifugation (13) prior to resuspension in 0.1M sodium phosphate-20% glycerol pH 7.4 at a protein concentration of 15-20mg/ml and stored in 0.1ml aliquots at -80°C.

Enzyme Assays

All assays used a freshly thawed aliquot of microsomes and were carried out within 2 weeks of isolation.

Cytochromes P-450 and b_5 were determined by difference spectroscopy (14). Hexabarbital demethylation was determined by a formaldehyde release assay (15,16) and AHH activity from the rate of 3-hydroxylation of benzo[α]pyrene (17). Aniline hydroxylation was assayed by p-aminophenol production (18). Cytochrome P-450 reductase (19) and cytochrome b_5 reductase (20) activities were determined by the NADPH and NADH dependent reduction of ferricyanide respectively, in the presence of 0.25mM KCN. Epoxide hydrase activity was measured by conversion of p-nitrostyrene oxide to its diol and analysed by high pressure liquid chromatography (21).

Protein concentration was determined by the method of Lowry *et al.* (22) using crystalline Bovine albumin as a standard.

Gel Electrophoresis

Polyacrylamide gels were run essentially as described by Laemmli (23) using a 4% stack and 9% running gel, prior to silver staining of proteins by the method of Wray *et al.* (24).

Statistical Analysis

Data was compared using the Mann-Whitney non-parametric procedure (25).

RESULTS AND DISCUSSION

In preliminary experiments the induction response of the chick embryo with respect to total cytochrome P-450 was examined using a range of 2AAF, PB and 3-MC dosages. The amounts used in this study maximised the total cytochrome P-450 induction (data not shown). There was no sex difference for any activity measured in control or treated embryos (data not shown); the data presented here is thus pooled from microsomal preparations derived from animals of both sexes.

Induction of Cytochrome P-450 and Monooxygenase Activities

PB and 3-MC induce P-450 in chick embryos 5-fold and 2-fold respectively (Table 1). A 3-fold induction of cytochrome P-450 by 2AAF was observed; this contrasts with the small inducing effect (40%) observed in the rat (1). This induction by 2AAF in the chick embryo has been achieved reproducibly

TABLE 1

Microsomes	Cytochrome P-450[a]	AHH[b]	Hexabarbital[c] demethylation	Aniline[d] hydroxylation	Aniline hydroxylation + 0.1mM Metyrapone
Control	177 ± 12	51 ± 4	1.30 ± 0.13	17.5 ± 0.4	7.5 ± 0.2
PB.	908 ± 122*** (5.1)	133 ± 24*** (2.7)	9.80 ± 0.80*** (7.4)	86.5 ± 9.9*** (4.9)	46.3 ± 2.3
3-MC.	378 ± 29*** (2.1)	521 ± 73*** (10.2)	2.60 ± 0.20*** (2.0)	50.0 ± 2.9*** (2.9)	39.8 ± 3.1
2AAF.	605 ± 73*** (3.4)	137 ± 18*** (2.7)	9.85 ± 1.20*** (7.4)	48.0 ± 0.4*** (2.7)	25.6 ± 0.2

Assays were carried out as described in "Materials and Methods". Values are means ± S.D from duplicate assays on 6-8 preparations. Enzyme activity units are : a, pmol mg^{-1} protein : b, pmol 3-OH benzo[α]pyrene min^{-1} mg^{-1} protein : c, nmol formaldehyde min^{-1} mg^{-1} protein : nmol p-aminophenol hr^{-1} mg^{-1} protein.
*$p<0.05$; ***$p<0.01$; ***$p<0.001$; using Mann-Whitney Test.

over a period of 12 months using 2AAF supplied from two commercial sources (data not shown).

Individual monooxygenase activities are increased to differing extents by the three agents (Table 1). PB and 2AAF elicit a 7.4-fold increase in hexabarbital demethylation but only increase AHH activity 2.7-fold. This is the reverse of 3-MC which produces a 10-fold increase in AHH activity and a 2-fold increase in hexabarbital demethylation. These results suggest some similarity between the induction by PB and 2AAF which is supported by the characteristics of metyrapone inhibition upon aniline hydroxylation; metyrapone in the rat shows a higher affinity for PB inducible P-450 than 3-MC inducible P-450 (26). 2AAF and PB induced chick embryo microsomes have a similar sensitivity to inhibition of aniline hydroxylation by metyrapone (49% and 47% respectively) whilst the activity of 3-MC induced microsomes is only reduced by 19% (Table 1).

Electrophoresis of Microsomes

The similarity between 2AAF and phenabarbitone induction extends to the profile of induced proteins observed after electrophoresis (Fig.1). PB and 2AAF induce bands "a" and "b" whilst 3-MC induces the higher molecular weight band "c". The higher resolution of low protein loading coupled with silver staining reveals differences between PB and 2AAF with the induction of bands "d" and "e" respectively.

Effects Upon Other Microsomal Enzymes Involved in Xenobiotic Metabolism

Compared to changes in cytochrome P-450 and monooxygenase functions caused by these agents there are only relatively small changes in other enzyme activities (Table 2). Although PB and 2AAF are inducers of p-nitrostyrene oxide hydration by rat liver microsomes, presumably due to increased epoxide hydrase (data not shown). No increase could be produced in chick embryo. Previous data is not available on chick embryo epoxide hydrase or its induction; in neonatal animals activity is lower than in the adult and difficult to induce (27). The 19% reduction in epoxide hydrase activity

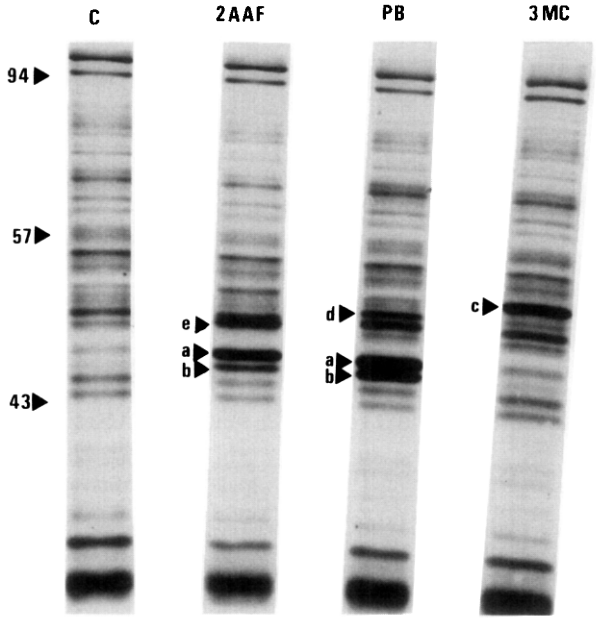

Fig.1. Electrophoresis and staining of microsomal protein carried out as described in "Materials and Methods".

($p<0.01$) we observed in microsomes prepared from 3-MC induced animals may be due to contamination with (inhibitory) metabolites of this agent. In fact 3-MC metabolites have been used as substrates for epoxide hydrase (28).

2AAF has no significant effect ($p>0.05$) on other microsomal electron transport activities in the chick embryo; this contrasts to its induction of cytochrome b_5 and cytochrome P-450 reductase in rat (1). Induction of electron transport enzymes occurs after administration of PB to the chick

TABLE 2

Microsomes	Cytochrome b5 [a]	Epoxide hydrase [b]	Cytochrome b5 reductase [c]	Cytochrome P-450 reductase [c]
Control	112 ± 15	3.2 ± 0.2	1.56 ± 0.20	0.46 ± 0.06
PB.	162 ± 13*** (1.4)	2.8 ± 0.3 (0.9)	1.04 ± 0.07*** (0.7)	0.80 ± 0.07*** (1.7)
3-MC.	121 ± 8 (1.1)	2.6 ± 0.3** (0.8)	1.30 ± 0.15* (0.8)	0.46 ± 0.08 (1.0)
2AAF.	117 ± 15 (1.0)	2.8 ± 0.4 (0.9)	1.70 ± 0.30 (1.1)	0.49 ± 0.03 (1.0)

Enzyme activities are : a, pmol mg^{-1} protein : b, nmol p-nitrostyrene diol min^{-1} mg^{-1} protein : c, µmol ferricyanide min^{-1} mg^{-1} protein. All other details as table 1.

embryo with a 1.7 fold increase in cytochrome P-450 reductase ($p< 0.001$) and a 1.4 fold increase in cytochrome b_5 ($p<0.001$); this is a point of dissimilarity with 2AAF treated embryos where no such changes occur. The reduction in the specific content of cytochrome b_5 reductase during phenabarbitone induction in the chick has been previously noted in the rat (29).

CONCLUSIONS

Avian species are relatively resistant to 2AAF induced carcinogenesis requiring higher doses and longer periods than rodents (10). Chicks are capable both of aromatic and N-hydroxylation of 2AAF which in the rat are catalysed by microsomal cytochrome P-450s (4-7,30). Administration of cytochrome P-450 inducing agents may increase oxidative metabolism of 2AAF and shift the balance between detoxification (aromatic hydroxylation) and activation (N-hydroxylation). 3-MC increases the rate of aromatic hydroxylation in the rat to a greater extent than it increases N-hydroxylation, thus reducing the proportion of 2AAF activated and imparting a resistance to hepatocarcinogenesis (30).

We are currently investigating whether 2AAF induces its own metabolism in the chick embryo in a similar manner to account in part for avian resistance to this agent. We do not however exclude the possibility that this may be due to some property of the complex phase II metabolism of this compound (7).

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