Biochemical Pharmacology, Vol. 30, No. 11, pp. 1197–1201, 1981.
Printed in Great Britain.

0006-2952/81/111197-05 $02.00/0
Pergamon Press Ltd.

THE EFFECT OF AROCLOR 1254 PRETREATMENT ON THE PHASE I AND PHASE II METABOLISM OF 7-ETHOXYCOUMARIN IN ISOLATED VIABLE RAT KIDNEY CELLS

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(*Received* 4 *August* 1980; *accepted* 14 *November* 1980)

Abstract—The metabolism of 7-ethoxy- and 7-hydroxy-coumarin was studied in viable kidney cortex cells isolated from control and Aroclor 1254-pretreated rats. Such pretreatment led to induction of the microsomal mono-oxygenase-mediated Phase I system but did not produce induction of Phase II glucuronidation or sulphation activity. Induction of the microsomal mono-oxygenase system led to an increase in the amount of unconjugated Phase I metabolite present during sequential Phase I and Phase II metabolism. It is suggested that this increase is due to some form of 'uncoupling' of the microsomal mono-oxygenase and glucuronidation systems.

There is increasing awareness that the nephrotoxicity of a number of organic chemicals is mediated through the *in situ* production of reactive metabolites [1–4]. It is also becoming apparent that the balance of activation pathways of metabolism (usually Phase I reactions) versus inactivation pathways of metabolism (usually Phase II reactions) is of importance in many examples of metabolism-mediated nephrotoxicity [1].

Most of the drug metabolizing capability of mammalian kidney resides in the cortex region [5, 6]. Recent evidence indicates that, at least in rabbit kidney, oxidation of xenobiotics in the cortex proceeds via the cytochrome P-450-mediated microsomal mono-oxygenase system whereas similar oxidation in the medulla is mediated via a prostaglandin co-oxidation mechanism [7]. Under control conditions (i.e. in non-induced animals) the Phase I metabolic capability of kidney cortex is low relative to that in liver [5, 8] whereas the Phase II metabolic capability of the cortex approaches that in liver [8, 9]. However, induction of the MMO system† leads to a great increase in the level of Phase I metabolism in the cortex [10–13], and this increase in the level of Phase I metabolism has been associated with modification of xenobiotic-mediated nephrotoxicity [3, 4]. The effect of inducers of the MMO system on renal Phase II metabolism appears to have been little studied although a report by Aitio [14] indicates that rat renal UDP-glucuronyltransferase activity is unaffected after treatment with either 3-methylcholanthrene or phenobarbitone (two commonly used inducers of the hepatic MMO system) even though hepatic UDP-glucuronyltransferase activity was well induced by such treatments.

Recently, Jones *et al.* [15] have reported on the metabolism of paracetamol to glucuronic acid, sulphate and sulphydryl conjugates in isolated rat kidney cells. The production of sulphydryl conjugates of paracetamol is believed to occur subsequent to generation of a reactive Phase I metabolite, and in this context it is of interest to note that pre-treatment of rats with 3-methylcholanthrene greatly increased the rate of formation of sulphydryl conjugates together with a smaller increase in the rate of glucuronide formation, the rate of sulphation being unaltered [15]. However, it was not possible to directly and separately measure the amounts of sequentially-produced Phase I and Phase II metabolites using this system. We have therefore studied the sequential metabolism of a model xenobiotic (7-ethoxycoumarin) in viable rat kidney cortex cells isolated from control and induced animals. A previous study has demonstrated that 7-EC is metabolized by isolated viable rat kidney cortex cells to 7-hydroxycoumarin which is subsequently conjugated with glucuronic acid and sulphate [8]. The enzyme inducer chosen for this study was Aroclor 1254, a mixture of polychlorinated biphenyls, which is an ubiquitous environmental pollutant [16, 17] and is known to be a potent inducer of the renal MMO system [11].

MATERIALS AND METHODS

Animals and treatment. Male Wistar rats (90–100 g), locally-bred, were used in these studies. The animals were allowed food (rat diet 41B, Wherry and Son, Bourne, England) and water *ad libitum*, and they were housed in wire-bottomed cages (2 animals per cage) kept in a room on a 12 hr light/dark cycle with the temperature maintained at 22–23°.

Aroclor 1254 (diluted in Arachis oil to 200 mg/ml) was injected intraperitoneally at a dose of 500 mg/kg. Control animals received an appropriate volume of the Arachis oil. The animals were killed five days after injection.

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† Abbreviations used: 7-EC, 7-ethoxycoumarin; 7-HC, 7-hydroxycoumarin; MMO, microsomal mono-oxygenase.

Cell isolation. Viable kidney cortex tubules were isolated by collagenase dissociation of cortex slices as described previously [8, 9] with the modification that the collagenase solution was buffered to pH 7.4 with 10 mM HEPES, and the tubules isolated from one pair of kidneys were finally resuspended in 5 ml L15 culture medium containing 10% (v/v) calf serum. Cell viability was assessed by a trypan-blue exclusion method as described previously [8, 9]. Viabilities of greater than 90 per cent were obtained from control and induced rats.

Assays. Samples (3 ml) of the tubule suspensions were incubated in 25 ml Erlenmeyer flasks with shaking at 37° under room air in the presence of either 7-ethoxycoumarin (100 μM) or 7-hydroxycoumarin (200 μM) for up to 2 hr. At various times 0.5 ml samples were removed and diluted in 3.0 ml ice-cold distilled water prior to analysis. The substrates were added in 6 μl dimethyl-formamide. The extraction and measurement of metabolites and the enzymic hydrolysis of the conjugated metabolites was carried out as described previously [8]. Cellular protein was assayed by the method of Lowry *et al.* [18] following centrifugation of a sample of the cell suspension, disruption of the resultant cell pellet with 1% aqueous Triton X-100 and digestion in 0.5 N NaOH. Hepatic cytochrome P-450 content was assayed on samples of liver homogenate by the method of Joly *et al.* [19].

The sources of chemicals, enzymes and culture media have been described previously [8, 9]. Aroclor 1254 was obtained from the Monsanto Chemical Co., St. Louis, MO, U.S.A.

Analysis and presentation of results. Results from 4–8 rats are expressed as mean ± S.D or as mean ± range. Because of the inter-animal variation in some of the enzymes activities (see Results) statistical evaluation of the results was performed by use of the Mann–Whitney U-test [20]. The levels of unconjugated 7-HC, 7-HC glucuronide and 7-HC sulphate were measured separately, and a value for the total 7-HC produced was obtained by summation of these separate values.

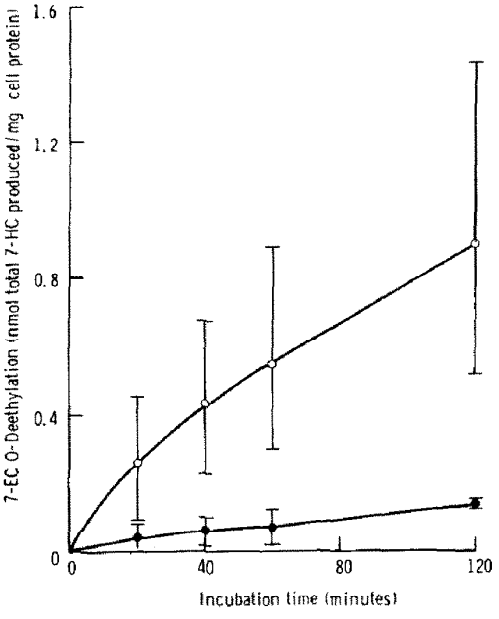

Fig. 1. Time dependence of 7-EC *O*-deethylation in viable kidney cortex cells isolated from Arachis oil and Aroclor 1254-pretreated rats. Cells were incubated with 100 μM 7-EC and the total production of 7-HC assayed at various times as outlined in the text. Each point is the mean value of 4–8 rats, the vertical bars indicating the range of values. Symbols: ●, Arachis oil-pretreated rats; ○, Aroclor 1254-pretreated rats. Values for the Aroclor 1254-pretreated rats were significantly different (at P = 0.01) from the Arachis-oil pretreated rats at all time points.

RESULTS

The time course of 7-EC *O*-deethylation by viable cortex cells isolated from control and induced rats is shown in Fig. 1. Cells isolated from control rats have a low but detectable deethylase activity of approx. 3 pmoles total 7-HC produced/mg cell protein/min (Table 1) and by 120 min incubation only

Table 1. Comparison of yield and enzyme activities of viable kidney cortex cells isolated from control or Aroclor 1254-pretreated rats

	Control	Aroclor 1254
Kidney cell yield (mg protein/ml suspension)	3.925 ± 1.110(16) N.S.	4.037 ± 1.656(16)
7-EC *O*-Deethylase activity	2.90 ± 1.74(8) P < 0.01	18.62 ± 12.53(8)
7-HC Glucuronidation activity	160.40 ± 42.04(4) N.S.	131.20 ± 37.73(4)
7-HC Sulphation activity	12.75 ± 2.94(4) N.S.	13.42 ± 5.17(4)

Animal pretreatment, cell isolation and assays were performed as outlined in the text. All the results are mean ± S.D. with the number of animals studied given in brackets. Enzyme activity expressed as pmoles product formed/mg cell protein/minute. Statistical evaluation was performed using the Mann–Whitney U-test (N.S. indicates no significant difference between the two groups at the P = 0.05 level). Glucuronidation activity was measured as maximal rate (see Fig. 2). Other enzyme activities measured as initial rates.

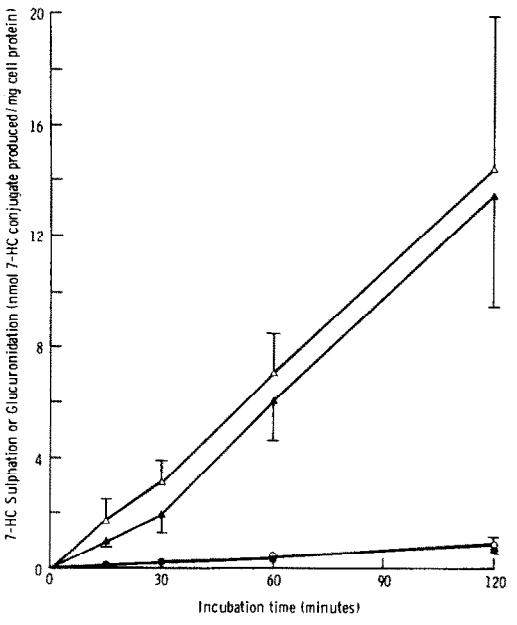

Fig. 2. Time dependence of 7-HC conjugation with glucuronic acid and sulphate in viable kidney cortex cells isolated from Arachis oil- and Aroclor 1254-pretreated rats. Cells were incubated with 200 μM 7-HC and the production of 7-HC conjugates assayed at various times as outlined in the text. Each point is the mean ± S.D. of 4 rats in each group. Symbols: △, ▲ conjugation of 7-HC with glucuronic acid; ○, ● conjugation of 7-HC with sulphate; Open symbols: Arachis oil-pretreated rats; Closed symbols: Aroclor 1254-pretreated rats. There was no significant difference (at $P = 0.05$) between the two groups (for either conjugate) at any time point.

0.6 per cent of the 7-EC had been converted to 7-HC. Treatment of the rats with Aroclor 1254 greatly increased the extent of deethylation (3.6% conversion at 120 min) (Fig. 1) and the deethylase activity increased to 18.6 pmoles total 7-HC produced/mg cell protein/min, an increase of 520 per cent above control. This induction of renal MMO activity was paralleled by similar induction of hepatic cytochrome P-450 content (data not shown). A consistent feature that emerged from these experiments was the marked inter-animal variability in both the extent of metabolism and the specific enzyme activity, particularly in cells isolated from induced rats (Fig. 1, Table 1). This large inter-animal variability was not due to variation in amount of inducer administered (as judged by a lack of similar variation in the hepatic cytochrome P-450 content), neither was it due to an alteration in the efficiency of enzymic isolation of the cells (protein content of cells isolated from control and induced animals were similar (Table 1)) or alteration in cell viability (trypan blue exclusion similar in control and induced situations). Similar large inter-animal variability in certain renal MMO-mediated reactions has been reported in previous microsomal studies [13, 21–25] and it is possible that the kidney MMO system is much more sensitive to the modulating effects of endogenous agents than is the hepatic system, as has been suggested by Van Cantfort and Gielen [24].

The influence of Aroclor 1254 pretreatment on the direct conjugation of 7-HC with glucuronic acid and sulphate was next studied. In renal cortex cells isolated from both control and induced rats, conjugation with glucuronic acid was easily the major route of conjugation, sulphate conjugation accounting for only 5% per cent of the total conjugation (Fig. 2). Pretreatment of the rats with Aroclor 1254 resulted in no significant alteration in the pattern or extent of 7-hydroxycoumarin conjugation and the specific maximal enzyme activities were similar in the two groups of rats (Table 1).

Having established the effects of Aroclor 1254 pretreatment on the overall renal conversion of 7-EC to 7-HC and the direct conjugation of 7-HC, the last series of experiments were designed to study the effects of Aroclor 1254 pretreatment on the integrated (i.e. Phase I and Phase II) metabolism of 7-EC. The results are illustrated in Fig. 3, each point

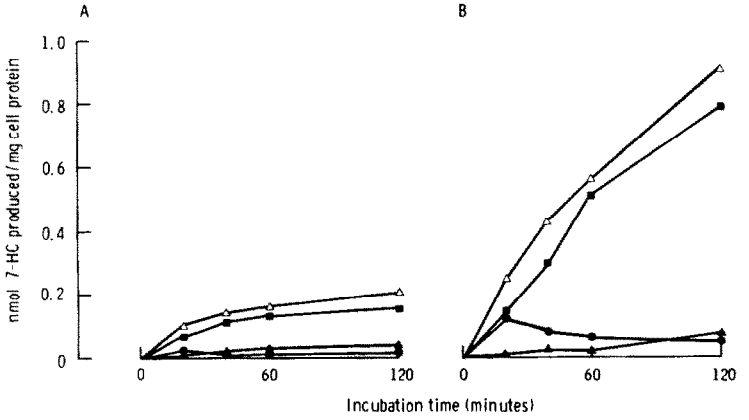

Fig. 3. Pattern of 7-EC metabolism in viable kidney cortex cells isolated from (A) Arachis-oil, and (B) Aroclor 1254-pretreated rats. Cells were incubated with 100 μM 7-EC and the amount of unconjugated 7-HC (●), 7-HC sulphate (▲) and 7-HC glucuronide (■) present at various time points was measured. The amount of total 7-HC (△) present at each time point was obtained by summation of the amounts of these three metabolites. Each point is the mean value derived from 4 rats.

on the graph indicating the mean value of four animals with the S.D. omitted due to the large inter-animal variability mentioned previously. However, the pattern of 7-EC metabolism was similar in all animals of either group.

In renal cortex cells isolated from control rats 7-EC metabolism had stopped by 120 minutes' incubation (Figs 1 and 3) and throughout this time period 7-HC glucuronide was the major metabolite. Unconjugated (i.e. free) 7-HC was a minor metabolite (<20%) throughout the 120-min incubation period. This would be expected from the low level of deethylase activity relative to that for conjugation.

In renal cortex cells isolated from Aroclor 1254–pretreated rats 7-EC metabolism was still progressing at 120 min incubation (Fig. 3) with 7-HC glucuronide once again being the major metabolite. At the end of the incubation period the amount of 7-HC glucuronide present in the induced cell incubation had increased by almost 400 per cent relative to that present in the control incubations. The level of sulphate conjugation of 7-hydroxycoumarin was essentially unchanged by the Aroclor pretreatment. At 20 min of incubation unconjugated 7-HC was a major metabolite accounting for approximately 40 per cent of the total metabolites at that time point (compared with 20% in the control situation) but after this time its level decreased although there was considerably more unconjugated 7-HC present throughout the entire incubation period in the induced situation than in the control situation (Fig. 3).

DISCUSSION

The results of this study clearly demonstrate that renal MMO activity is well induced by Aroclor 1254 pretreatment but that glucuronidation and sulphation activity are not induced by this pretreatment. Similar findings have been observed using 3-methylcholanthrene as the inducer (J. R. Fry, unpublished work). This is broadly in agreement with the findings of Jones *et al.* [15]. Interestingly, there appears to be a substrate difference in inducibility of the renal glucuronidation pathway, paracetamol glucuronidation being inducible [15] whereas 7-HC glucuronidation is not inducible (this paper). Whether this indicates the existence of different renal UDP-glucuronyltransferases is at present unknown.

Increasing the amount of 7-HC produced from 7-EC (as in induction) also increased the subsequent glucuronidation of 7-HC but not its sulphation (Fig. 3), thus indicating that the renal sulphation pathway is readily saturable at low substrate concentrations. Similar findings have been observed in isolated rat hepatocytes [25].

A potentially important finding of this study was that induction of the kidney MMO system led to an increase in the amount of unconjugated Phase I metabolite present during the early stages of consecutive Phase I/Phase II metabolism. Previous studies with isolated rat hepatocytes have demonstrated that one explanation for an increase in the level of unconjugated Phase I metabolite on induction is that of a lag in glucuronidation of Phase I metabolite which precedes activation of this pathway [25]. This 'lag-activation' phenomenon does not appear to be operating in the present situation however, since the production of 7-HC glucuronide was linear throughout the first 20 min of incubation (data not shown). Rather it is probable that this increase in the level of unconjugated Phase I metabolite on induction is due to some form of uncoupling of the Phase I MMO reaction and the subsequent Phase II glucuronide conjugation. Previous hepatocyte studies have demonstrated that there is no obligatory coupling of Phase I MMO activity and Phase II glucuronidation, both of which are located on the endoplasmic reticulum but that under normal control conditions the two systems act in concert. However, it is possible to experimentally inhibit the Phase II reactions without influencing the preceding Phase I reaction [26]. The mechanism by which the uncoupling noted in the present study is brought about is uncertain, but may be a consequence of the induction process or the greatly increased rate of production of Phase I metabolite.

It is possible that this increase in the level of unconjugated Phase I metabolite that occurs on induction of the renal MMO system may be of toxicological significance, particularly as induction of the renal MMO system is not paralleled by induction of the renal glucuronidation system.

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