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PHASE 1 AND PHASE 2 DRUG METABOLISM IN ISOLATED EPIDERMAL CELLS FROM ADULT HAIRLESS MICE AND IN WHOLE HUMAN HAIR FOLLICLES

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Abstract—A sensitive fluorimetric assay to determine both Phase 1 (oxidation) and Phase 2 (conjugation) drug metabolism in epidermal cells isolated from hairless mice, using ethoxycoumarin as a model substrate, is described. Ethoxycoumarin was metabolized by isolated epidermal cells via dealkylation to 7-hydroxycoumarin (7-OHC) and subsequent conjugation. Phase 1 metabolites were extracted in ether from the aqueous incubation media, back extracted into sodium hydroxide and determined fluorimetrically. Conjugated metabolites remaining in the aqueous phase were hydrolysed by the action of β-glucuronidase and extracted and determined in a similar manner. The production of free 7-OHC by isolated epidermal cells was biphasic at all substrate concentrations tested, exhibiting an initial linear increase followed by a plateau phase. The plateau phase was attributable to the conjugation of 7-OHC produced *in situ*. Metabolism was inhibited by SKF 525A, carbon monoxide, and α-naphthoflavone. Endogenous supplies of reducing equivalents in the form of NADPH were adequate to attain maximal rates of metabolism. With human hair follicles both Phase 1 and Phase 2 activity was detectable in 7 out of 11 subjects. The assay has the advantages of being sensitive, producing single defined metabolites from both Phase 1 and Phase 2 metabolism; is readily adaptable to human skin samples.

Although sub-cellular fractionation followed by *in vitro* enzyme assay is widely used in the study of drug metabolizing enzymes [1], the conclusions that can be drawn from such studies are limited, particularly when attempting to extrapolate results obtained to the *in vivo* situation [2]. The rate of metabolism of a drug *in vivo* is influenced by factors such as heterogeneity of cell populations, cellular uptake of substrate to the active site of the enzyme, availability of cofactors or further metabolism of primary metabolites, none of which apply *in vitro* with enzyme preparations. In addition, the need to perform *in vitro* enzyme studies under strictly kinetic conditions of high substrate and cofactor concentrations can affect the stability of membrane bound drug metabolizing enzymes. Furthermore, the metabolism of a drug *in vivo* frequently proceeds via oxidation (Phase 1 metabolism) and subsequent conjugation (Phase 2 metabolism) of the oxidized substrate [3]. The use of sub-cellular fractions in *in vitro* enzyme assays does not afford any information on the sequential metabolism of a drug substrate in this way.

In attempts to overcome these limitations, and attain an *in vitro* assay system that more closely resembles the *in vivo* situation, a variety of techniques using isolated cell preparations or organ perfusion have been developed, particularly for the liver and the gut [4–6, 2]. For drug metabolism in the skin most of the studies done so far have used either whole tissue homogenates or sub-cellular fractions [7, 8]. However, because of the unique properties and function of the skin the limitations of *in vitro* sub-cellular fractionation procedures are of particular relevance. The skin is a heterogeneous mixture of many different cell populations and inert structural proteins. Activity may differ between the former whilst the latter may be reflected in a low specific activity of cutaneous drug metabolism in relationship to unit of mass or protein. In addition, the harsh homogenization techniques required to disrupt cutaneous tissue have deleterious effects on the activity of drug metabolizing enzymes [9]. The relatively low yields of microsomal protein obtained from skin (approximately 10-fold less than the liver [10, 11]), and the intrinsically low activity of drug metabolizing enzymes in the skin are also a problem in *in vitro* assay systems, particularly with human skin [11]. Recently Moloney *et al.* [12] have reported that ethoxycoumarin is metabolized by rat and hairless mouse skin strips, *in vitro*, by dealkylation to 7-hydroxycoumarin and subsequent conjugation of 7-hydroxycoumarin to its glucuronide. Furthermore ethoxycoumarin dealkylation in the skin has been shown to be inducible by both polycyclic hydrocarbons and phenobarbitone [13]. Ethoxycoumarin thus appears to be a particularly useful substrate for the study of mono-oxygenase activity in the skin. The present study was initiated to develop an *in vitro* isolated cell assay for cutaneous drug metabolism which overcame the limitations inherent in the more frequently used *in vitro* assay systems, and which was applicable to human skin samples.

MATERIALS AND METHODS

Hanks balanced salt solution (Solution A); calcium and magnesium free Hanks balanced salt solution (Solution B) and trypsin (1:250) were obtained from

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Gibco Ltd. (Paisley, Scotland). Deoxyribonuclease (DNase) Type 1; sulfatase Type H-5 containing 400,000 units of glucuronidase activity per ml; and glucuronidase Type B-10 were purchased from Sigma (London). Ethoxycoumarin and 7-hydroxycoumarin were purchased from the Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and recrystallized prior to use. All solvents used were of spectroscopic grade. Adult inbred hairless (hr/hr) mice bred in the departmental animal house were used for all expermments.

Separation and isolation of hairless mouse epidermal cells

Epidermis was separated from whole skin by a modification of the trypsin flotation procedure. Mice were killed by decapitation or cervical dislocation, the trunk skin removed, and sub-cutaneous fat and tissue removed by scraping with a scalpel. The skin was then cut into strips approximately 1 cm wide by 10 cm in length and placed dermal side down on Solution B containing 5 mM EDTA and 0.5% trypsin. After incubation overnight for 18 hr at 2–4° the strips of skin were rinsed thoroughly with Solution B. The skin pieces were then opened out on a flat surface and the epidermis peeled from the dermis using a blunt scalpel blade. Separated epidermal sheets were then placed in Solution A containing 2 mg/ml DNase and incubated for 15 min at room temperature with occasional agitation. The turbid supernatant was then aspirated using a Pasteur pippette and the remaining large residual pieces of epidermis discarded. Epidermal cells were harvested from the supernatant by centrifugation at 600 rpm for 5 min, washed and resuspended in Solution A to a final concentration of 10^6 cells/ml. Cell viability was assessed using trypan blue.

7-ethoxycoumarin metabolism

Epidermal cell suspensions (10–20 ml) were placed in conical flasks and incubated in a shaking water bath at 37°. The reaction was started by the addition of various concentrations of substrate (see text) dissolved in methanol/water (25% v/v). After incubation for various time periods 2 ml aliquots of the reaction mixture were removed and added to 3 ml of *n*-hexane in an ice bath. Unchanged 7-ethoxycoumarin (EOC) was extracted from the aqueous phase using three sequential *n*-hexane extractions of 3 ml. The *n*-hexane extracts were discarded and the remaining aqueous phase then extracted twice with diethyl ether (2 × 4 ml). The 2 × 4 ml diethyl ether layers containing the 7-hydroxycoumarin (7-OHC) were pooled and then back extracted into 0.5 ml of 0.01 M NaOH/1.0 M NaCl. The concentration of 7-OHC in 0.3 ml of the 0.01 M NaOH/1.0 M NaCl phase was determined fluorimetrically. Conjugated metabolites remaining in the aqueous phase after ether extraction were hydrolysed by overnight incubation with sulfatase or β-glucuronidase at 37°, and extracted in an identical manner. Similar results were obtained with both sulfatase and β-glucuronidase as enzyme, although blank values obtained with β-glucuronidase were lower. 7-OHC was quantified fluorimetrically at excitation and emission wavelengths of 368 nm and 456 nm respectively [14]. Blanks for the assay were either zero time controls or cells that had been lysed by suspending in distilled water for 15 min. In some experiments total metabolites were determined by removing the cells from the incubation media by centrifugation, adjusting the pH to 5.0 with 0.5 M citrate buffer and incubating overnight with β-glucuronidase. The pH was then adjusted to 1.5 by the addition of trichloroacetic acid (1.25% w/v) and metabolites extracted in 3 ml of chloroform. The aqueous layer was discarded and 2 ml of the chloroform phase extracted with 0.5 ml of 0.01 M NaOH/1.0 M NaCl. The chloroform layer was discarded and the concentration of 7-OHC in the alkaline aqueous layer estimated fluorimetrically.

Human hair follicles

Between 40 and 80 hairs were plucked from 11 healthy volunteers (5 female). The crown of the head was avoided in balding males. The hair bulbs were placed in 2 ml of solution A containing 0.1 mM EOC and incubated for 4–6 hr at 37°. Metabolites were extracted and quantified as described above. DNA content was determined using the mithramycin binding assay as previously described [15].

RESULTS

The effect of EOC on the viability of epidermal cells

The viability of epidermal cells isolated using trypsin/DNase was 86 ± 12% (N = 25) with viable cells representing all epidermal cell types. Direct trypsinization of cells from epidermal sheets at room temperature or at 37°, gave reduced yields of viable cells (<20%) and totally destroyed drug metabolizing activity.

In the conventional microsomal enzyme assay of EOC dealkylation the concentration of substrate used is 0.5 mM [14]. Incubation of isolated epidermal cells with this concentration of EOC resulted in a marked decrease in cell viability (Fig. 1). This

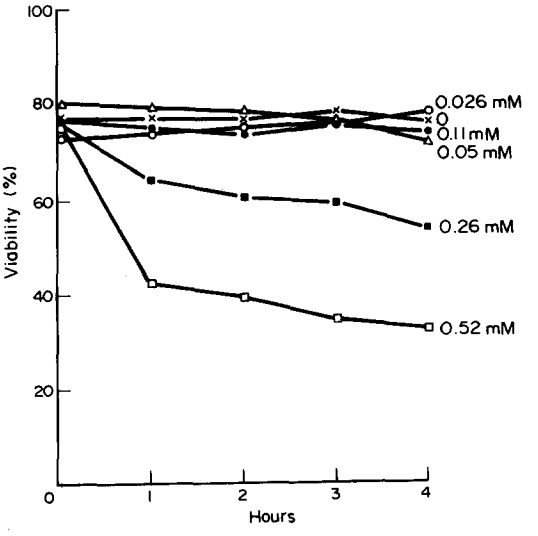

Fig. 1. The effect of 7-ethoxycoumarin on the viability of isolated epidermal cells. Each point represents the mean of 4 separate experiments, with a standard deviation of less than 10%.

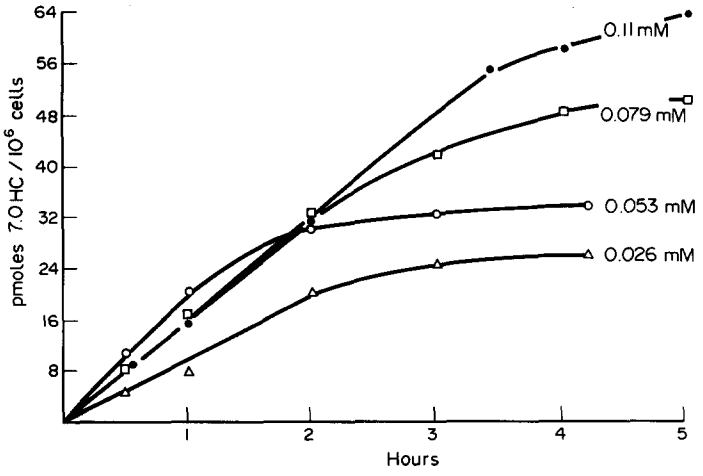

Fig. 2. Time course of the production of 7-hydroxycoumarin from 7-ethoxycoumarin by isolated epidermal cells. Each point represents the mean of 5 separate experiments, with a standard deviation of 12% or less.

decrease in viability was not observed for concentrations of EOC in the range of 5 μM to 0.1 mM although a small decrease in viability at 0.1 mM was evident at longer incubation times. Incubation times and substrate concentrations for subsequent metabolism studies were therefore chosen to minimize the decrease in cell viability at higher concentrations of EOC.

The metabolism of EOC by isolated mouse epidermal cells

The time course for the production of 7-OHC from EOC by isolated epidermal cells is shown in Fig. 2. At all the substrate concentrations tested the rate of production of free 7-OHC showed an initial linear increase, followed by a plateau phase during which the increase in the rate of formation of free 7-OHC decreased. The duration of the initial linear phase of 7-OHC production was dependent on the concentration of substrate used. At the higher substrate concentrations the initial linear phase was longer. The data presented in Fig. 3 indicate that the plateau phase in the production of free 7-OHC is due, at least in part, to the conjugation of 7-OHC. The production of conjugated 7-OHC from EOC became detectable at between 1 and 2 hr of incubation, at which time the production of free 7-OHC began to plateau. The time course for the production of conjugated derivatives of 7-OHC from 7-OHC produced *in situ*, shows a considerable lag phase during which no detectable conjugates were formed. An equivalent lag phase in the conjugation of 7-OHC added directly to isolated cells was not detectable at concentrations of 7-OHC from 5 μM to 0.1 mM (Fig. 3). Production of conjugated 7-OHC from EOC also reached a plateau phase at between 3 and 4 hr (Fig. 3). The most likely cause of this is substrate depletion since at this time up to 70% of the substrate had been metabolized. However, increased substrate concentration results in a sharp decrease in cell viability (Fig. 1).

The reproducibility of the assay (standard deviation of the mean) as determined by 20 replicate determinations of activity of a suspension containing 10^6 cells/ml was 6% for phase 1 metabolism and 14% for phase 2 metabolism.

Evidence for the involvement of cytochrome P-450 mediated reaction in dealkylation of EOC by epidermal cells is given in Table 1. The inhibitors of mono-oxygenase activity SKF 525-A, α-naphthoflavone and carbon monoxide all significantly inhibited the metabolism of EOC. EDTA did not

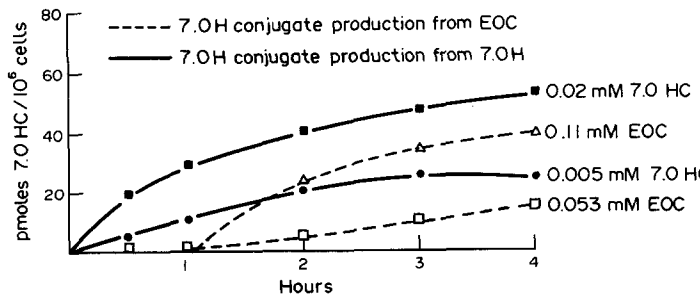

Fig. 3. Time course of the production of conjugated metabolites of 7-hydroxycoumarin from ethoxycoumarin (EOC) and 7-hydroxycoumarin (7-OHC). Each point represents the mean of 4 separate experiments with a standard deviation of less than 15%.

Table 1. The effects of mono-oxygenase inhibitors and NADPH on the production of 7-DHC by isolated epidermal cells

In vitro addition	7-OHC production % of control
None	100 (24 pmoles/hr/10^6 cells)
SKF 525A 10-6M	73 ± 12
αNF 10-6M	51 ± 6
CO	25 ± 10
NADPH 1.0 mM	110 ± 21
EDTA 1.0 mM	97 ± 17

Results which represent the mean of four experiments are shown as a percentage of the control with no *in vitro* additions.

inhibit the dealkylation of EOC. The addition of NADPH to isolated cell suspensions did not significantly increase the rate of dealkylation of EOC, indicating that endogenous supplies of reducing equivalents to the mono-oxygenase system are sufficient to attain maximal rates of metabolism.

EOC metabolism by human hair follicles

Having validated the assay using mouse epidermal cells, it was applied to human hair follicles. Phase 1 and phase 2 metabolism of EOC was detectable in 7 out of 11 subjects investigated (Table 2). Values varied over a 10-fold range and were not related to age, sex or smoking habits. Metabolism in human hair follices showed the same overall pattern of inhibition as mouse epidermal cells.

DISCUSSION

The advantages of isolated cell assay procedures over subcellular fractionation techniques in drug metabolism studies have recently been reviewed [2]. These advantages would appear to be particularly relevant in the study of drug metabolism in the skin, especially when attempting to identify those cutaneous cellular populations most active in drug metabolism. The use of EOC in the assay presently described has the advantages of producing well defined and unambiguous metabolites for both phase 1 and phase 2 reactions [12], in addition to being simple and reproducible. The method is also applicable to freshly isolated cells and human hair follicles without prior need for tissue culture. Fouts and his associates have reported the dealkylation of EOC by keratinocytes isolated by pronase or trypsin digestion [16, 17]. In agreement with our present findings they found the trypsinization at 37° resulted in a low yield of viable cells with a reduced drug metabolizing capacity. The pronase digestion gave similar yields to the trypsinization at 2–4° used presently. However, phase 2 metabolism of 7-OHC was not investigated. The present results show that certain similarities exist in the pattern of metabolism of drugs by isolated epidermal cells and isolated hepatocytes. The biphasic pattern of free 7-OHC production from EOC observed in the present study (Fig. 1) is similar to that reported for the production of 7-OHC and 2-hydroxy biphenyl by isolated hepatocytes [18]. These workers also noted an apparent lag between the hydroxylation of biphenyl and its subsequent conjugation, similar to that currently observed for 7-OHC in epidermal cells. Although the cause of this lag phase is uncertain it has been proposed that it is due to the activation requirements of UDP-glucuronyltransferase (UDP-GT) by small amounts of hydroxylated substrate [18]. The present data using 7-OHC would appear to support this assumption (Fig. 3). Alternatively the turnover number of UDP-GT at concentrations of substrate below its K_m may be too small for detection. However, the kinetics of sequential Phase 1 and Phase 2 metabolism in hepatocytes and epidermal cells appear similar. The observation that the supply of reducing equivalents in epidermal cells is sufficient to maintain maximal rates of mono-oxygenase activity is in keeping with the finding that NADP in the skin is predominantly in the reduced form [19]. The supply of NADPH for lipid synthesis in skin has been shown to be dependent to a large extent on malic enzyme, with the pentose phosphate pathway supplying only half of the required reducing equivalents [20] and it is probable that similar mechanisms operate with respect to drug metabolism in the skin.

Table 2. The metabolism of ethoxycoumarin by human hair follicles

		pmoles μg DNA/hr	
		Phase 1	Phase 2
1	AL	1.7	0.6
2	KS	3.2	1.4
3	JB	11.1	4.6
5	CF	7.3	1.6
6	MH	4.3	2.6
7	MF	1.2	0.9
8	RH	6.1	<0.1
9	PF	<0.1	<0.1
10	KR	<0.1	<0.1
11	SB	<0.1	<0.1
4	AA	<0.1	<0.1

With human hair follices activity was detectable in 7 out of 11 volunteers examined. EOC dealkylation in mice is under the same genetic control as NADPH dependent AHH mono-oxygenase activity [14]. It is possible therefore that this may be a reflection of genetic differences in EOD activity, although environmental influences are equally likely to result in such a variation.

Most previous studies on drug metabolism by intact skin cells and human hair folicles have been confined to the metabolism of polycyclic hydrocarbons in organ culture or cell culture [2, 21–23]. However, polycyclic hydrocarbons are not ideal substrates for the study of drug metabolism in cell preparations. The production of oxygenated metabolites of polycyclic hydrocarbons has been shown to occur independently of the drug metabolising enzymes by a number of biochemical or chemical mechanisms [24–29]. In view of the many mechanisms by which oxygenated BP metabolites can arise it is difficult to interpret results using BP as a substrate, particularly when taking measurements at the extreme limits of sensitivity, in tissues where the presence of mono-oxygenase activity has not been established. This is

particularly true of whole cell preparations in which prostaglandin synthetase, lipid peroxidation and hydroperoxidase will be active, and hydrogen peroxide generated through the action of numerous cellular reactions (e.g. glucose oxidase). In conclusion therefore, the assay we have described provides a reproducible and sensitive method isolating epidermal cells and for determining the activity of both Phase 1 and Phase 2 drug metabolising enzymes in isolated epidermal cells and human hair follicles. Work is currently in progress using this assay to define the particular cell types involved in drug metabolism in the skin.

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