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BRIEF COMMUNICATION

Comparison of the Metabolism of 7-Ethoxycoumarin and Coumarin in Precision-cut Rat Liver and Lung Slices

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Abstract—The metabolism of 7-ethoxycoumarin and [3-^{14}C]coumarin was compared in precision-cut rat liver and lung slices. The lung slices were prepared using an agarose gel instilling technique enabling the production of tissue cylinders followed by lung slices employing a Krumdieck tissue slicer. Both 50 μM 7-ethoxycoumarin and 50 μM [3-^{14}C]coumarin were metabolized by rat liver and lung slices. 7-Ethoxycoumarin was converted to 7-hydroxycoumarin (7-HC) which was conjugated with both D-glucuronic acid and sulfate. 7-HC sulfate was the major metabolite formed by both liver and lung slices. [3-^{14}C]Coumarin was metabolized by rat liver and lung slices to both polar products and to metabolite(s) that bound covalently to tissue slice proteins. The polar products included unidentified metabolites and 3-hydroxylation pathway products, with only very small quantities of 7-HC being formed. These results demonstrate that precision-cut lung slices are a useful model *in vitro* system for studying the pulmonary metabolism of xenobiotics. Moreover, the precision-cut tissue slice technique may be employed for comparisons of hepatic and extrahepatic xenobiotic metabolism.

Introduction

In recent years much effort has been devoted to the development of *in vitro* systems to study the metabolism and toxicity of xenobiotics. One very valuable *in vitro* model system is the use of precision-cut tissue slices, a technique developed by Krumdieck, Brendel, Gandolfi, Sipes and co-workers (Brendel *et al.*, 1987; Krumdieck *et al.*, 1980; Sipes *et al.*, 1987). With this technique, slices of uniform thickness may be prepared from the liver and other tissues and maintained in culture under appropriate conditions (Azri *et al.*, 1990; Brendel *et al.*, 1987).

The technique of precision-cut tissue slices has been applied to various tissues including the liver, kidney, lung and heart (Azri *et al.*, 1990; Parrish *et al.*, 1992; Ruegg *et al.*, 1989; Stefaniak *et al.*, 1992). With respect to the lung, an agarose gel instilling technique has been developed to enable tissue cores to be prepared from this relatively soft tissue (Placke and Fisher, 1987; Stefaniak *et al.*, 1992). Using this technique, precision-cut rat, mouse and human lung slices have been prepared and maintained in culture for periods up to 48 hr (Fisher *et al.*, 1994; Schwade and Thompson, 1994; Stefaniak *et al.*, 1992). The functional viability of cultured lung slices has been demonstrated by measurement of protein, DNA and phospholipid synthesis and their use for toxicity studies (Fisher *et al.*, 1994; Schwade and Thompson, 1994; Stefaniak *et al.*, 1992).

The aim of this study was to evaluate the usefulness of precision-cut lung slices as an *in vitro* model system for studies of xenobiotic metabolism. An additional objective was to ascertain whether the precision-cut tissue slice technique could be used for the comparison of hepatic and extrahepatic xenobiotic metabolism. In order to investigate both phase I and phase II pathways of xenobiotic metabolism, 7-ethoxycoumarin and coumarin were selected as model substrates. 7-Ethoxycoumarin is known to be metabolized by liver and lung preparations and is a substrate for cytochrome *P*-450 isoenzymes in the CYP1A, CYP2B and other subfamilies (Conney, 1986; Lake *et al.*, 1979; Vainio and Hietanen, 1980). In addition, 7-ethoxycoumarin has been used to study phase I and phase II xenobiotic metabolism in *in vitro* systems including precision-cut liver slices and

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Abbreviations: EBSS = Earle's balanced salt solution; 7-HC = 7-hydroxycoumarin.

hepatocytes (Barr *et al.*, 1991a,b; Paterson *et al.*, 1984). With coumarin, only hepatic metabolism appears to have been studied extensively (Cohen, 1979; Fentem and Fry, 1992; Lake *et al.*, 1992). Coumarin is known to be metabolized by purified rat hepatic CYP1A1 and CYP2B1 (Peters *et al.*, 1991), although it is also likely to be a substrate for other cytochrome *P*-450 isoenzymes in rat hepatic microsomes and in other species coumarin 7-hydroxylation is catalysed by CYP2A subfamily isoenzymes (Lake *et al.*, 1992; Pelkonen *et al.*, 1993).

Materials and Methods

Materials

The sources of the tissue culture materials, polystyrene incubation vials and stainless-steel wire mesh were as described previously (Beamand *et al.*, 1993; Lake *et al.*, 1993). [3-^{14}C]Coumarin (sp. act. 5.3 mCi/mmol) was obtained from ICI Chemicals and Polymers Group (Billingham, Cleveland, UK) and purified to greater than 99.5% radiochemical purity by chromatography on thin-layer plates of silica gel G developed in toluene–chloroform (1:1, v/v). Unlabelled coumarin metabolites were obtained from the sources described previously (Lake *et al.*, 1989). β-Glucuronidase (G-0251 from bovine liver), sulfatase (S-9626 from *Helix pomatia*) and D-saccharic acid 1,4-lactone were obtained from Sigma Chemical Co. (Poole, Dorset, UK), 7-ethoxycoumarin from Aldrich Chemical Co. (Gillingham, Dorset, UK) and SeaPrep agarose from Flowgen Instruments Ltd (Sittingbourne, Kent, UK).

Animals

Male Sprague–Dawley rats were purchased from Harlan Olac (Bicester, Oxon, UK) and allowed free access to R and M No. 1 diet (Special Diets Services, Witham, Essex, UK) and water. The animals were housed in mesh-floored cages in accommodation maintained at 22 ± 3°C with a relative humidity of 40–70%, and were allowed to acclimatize to these conditions for at least 2 wk before use. Rats (16–18 wk old) were killed by exsanguination under sodium pentobarbitone anaesthesia (60 mg/kg, ip). The livers and lungs were immediately excised and placed in Earle's balanced salt solution (EBSS) containing 25 mM D-glucose, 50 μg gentamicin/ml and 2.5 μg fungizone/ml previously gassed with 95% O_2/5% CO_2.

Preparation of liver slices

Tissue cylinders were prepared with a 10 mm diameter motor-driven tissue coring tool. From the cylinders, tissue slices (200–300 μm) were prepared in oxygenated (95% O_2/5% CO_2) EBSS containing the above additions at room temperature using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munford, AL, USA).

Preparation of lung slices

The trachea was cannulated and the lungs instilled with 0.75% (w/v) agarose solution at 37°C at a constant hydrostatic pressure of 20 cm as described previously (Placke and Fisher, 1987; Stefaniak *et al.*, 1992). After allowing the agarose to gel at 4°C, tissue cylinders were prepared with an 8 mm diameter motor-driven coring tool. From the cylinders, tissue slices (480–560 μm) were prepared in oxygenated (95% O_2/5% CO2) EBSS containing the above additions at 4°C using a Krumdieck tissue slicer.

Morphological investigations

Lung slices were fixed in neutral buffered formalin. Paraffin sections of about 5 μm thickness were cut and stained with haematoxylin and eosin. Lung slice thickness was measured with a VIDS IV Image Analysis System (Synoptics Ltd, Cambridge, UK).

Culture of tissue slices

Liver and lung slices were floated onto stainless-steel mesh inserts (two slices per insert) and cultured in polystyrene vials containing 1 ml culture medium employing a dynamic organ culture system as described previously (Beamand *et al.*, 1993; Lake *et al.*, 1993). The culture medium consisted of RPMI 1640 containing 5% foetal calf serum, 0.5 mM (final concentration) L-methionine, 1 μM insulin, 0.1 mM hydrocortisone-21-hemisuccinate, 50 μg gentamicin/ml and 2.5 μg fungizone/ml. The vials were placed on a roller system housed in a humidified incubator. They were incubated at 37°C in an atmosphere of 5% CO_2/95% air and rotated at approximately 9 rpm. After 30 min, treatment was commenced by replacing the culture medium with medium containing either 50 μM 7-ethoxycoumarin or 50 μM [3-^{14}C]coumarin dissolved in dimethyl sulfoxide (final concentration 0.5%, v/v). At the end of the incubation periods the medium was removed for analysis of metabolites (see below). Liver and lung slices were washed in 0.154 M KCl containing 50 mM Tris–HCl pH 7.4 and homogenized (two slices in 2 ml) in this medium by sonication (Beamand *et al.*, 1993). The liver and lung slice whole homogenates and media samples were stored at −80°C before analysis. Slice whole homogenate protein content was determined by the method of Lowry *et al.*, (1951), using bovine serum albumin as standard.

Metabolism of 7-ethoxycoumarin and [3-^{14}C]coumarin

Because previous studies have demonstrated that only small quantities of 7-ethoxycoumarin and polar [3-^{14}C]coumarin metabolites are retained in liver slices (Barr *et al.*, 1991a,b; Steensma *et al.*, 1994), only media samples were analysed for metabolites. The metabolism of 7-ethoxycoumarin to free 7-hydroxycoumarin (7-HC) and to 7-HC glucuronide and sulfate conjugates was determined by enzymatic hydrolysis and fluorimetric analysis of media samples

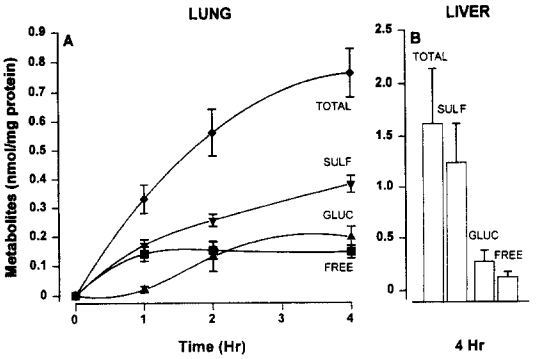

Fig. 1. Metabolism of 50 μM 7-ethoxycoumarin by precision-cut rat lung (A) and liver (B) slices. Lung slices were cultured for periods of 1, 2 and 4 hr and liver slices for 4 hr only and 7-ethoxycoumarin metabolism to unconjugated 7-HC (FREE), 7-HC glucuronide (GLUC), 7-HC sulfate (SULF) and total metabolism (TOTAL, i.e. free and unconjugated 7-HC) determined. Values are means ± SEM of three or four experiments.

incubated with 50 μM 7-ethoxycoumarin as described previously (Steensma *et al.*, 1994). The metabolism of [3-^{14}C]coumarin to polar metabolites was determined by enzymatic hydrolysis and HPLC separation of media samples incubated with [3-^{14}C]coumarin as described previously (Lake *et al.*, 1992; Peters *et al.*, 1991; Steensma *et al.*, 1994). Macromolecular binding of [3-^{14}C]coumarin metabolites to liver and lung slice proteins was determined by exhaustive solvent extraction (Lake, 1984; Lake *et al.*, 1992).

Results

Preparation and culture of rat lung slices

Morphological examination of freshly cut rat lung slices revealed a normal histological appearance (data not shown). Slice thickness, determined by examination of formalin-fixed slices, was 520 ± 18 μm (mean ± SEM of six experiments). Rat lung slices were cultured using a dynamic organ culture system under conditions previously employed for liver slices from the rat and other species (Beamand *et al.*, 1993; Lake *et al.*, 1993; Steensma *et al.*, 1994).

Metabolism of 7-ethoxycoumarin

7-Ethoxycoumarin is known to be deethylated to 7-HC, which can be conjugated with both D-glucuronic acid and sulfate (Barr *et al.*, 1991a,b; Paterson *et al.*, 1984; Vainio and Hietanen, 1980). A time-dependent metabolism of 50 μM 7-ethoxycoumarin was observed in precision-cut rat lung slices cultured for periods of 1, 2 and 4 hr (Fig. 1A). The major 7-ethoxycoumarin product was 7-HC sulfate, which accounted for some 51% of total metabolism after 4 hr, whereas free 7-HC and 7-HC glucuronide accounted for 21 and 28% of total metabolism, respectively. Compared with rat lung slices, total 7-ethoxycoumarin metabolism was 2.1-fold greater in rat liver slices after 4 hr (Fig. 1B). 7-HC sulfate was again the major product formed with free 7-HC, 7-HC glucuronide and 7-HC sulfate accounting for 6.5, 16.5 and 77% of total 7-ethoxycoumarin metabolism, respectively.

Metabolism of [3-^{14}C]coumarin

Coumarin is known to be metabolized by rat hepatic preparations (Cohen, 1979; Fentem and Fry, 1992; Lake *et al.*, 1992; Peters *et al.*, 1991; Steensma *et al.*, 1994) to various products including a 3-hydroxylation pathway which is followed by ring opening and further metabolism to other products including *o*-hydroxyphenyllactic acid, *o*-hydroxyphenylacetaldehyde, *o*-hydroxyphenylethanol and *o*-hydroxyphenylacetic acid. A time-dependent metabolism of 50 μM [3-^{14}C]coumarin to both polar products (Fig. 2A) and metabolite(s) that bound covalently to slice proteins (Fig. 2B) was observed in precision-cut rat lung slices cultured for periods of 1, 2 and 4 hr (Fig. 2B). Compared with rat lung slices, [3-^{14}C]-coumarin metabolism to total polar products and covalently bound metabolites was 1.2- and 2.9-fold greater, respectively, in rat liver slices after 4 hr (Fig. 2A,B). In keeping with previous studies with rat liver slices (Steensma *et al.*, 1994), the major metabolites of coumarin in rat liver slices in this study and

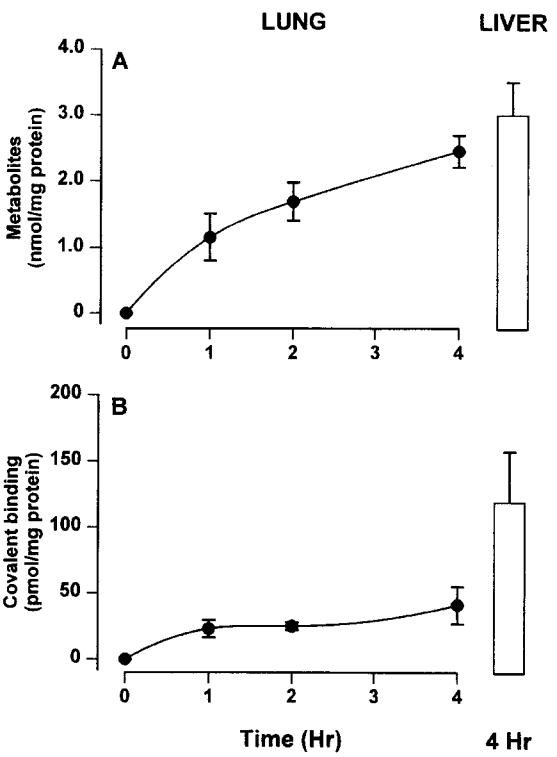

Fig. 2. Metabolism of 50 μM [3-^{14}C]coumarin by precision-cut rat lung and liver slices. Lung slices were cultured for periods of 1, 2 and 4 hr and liver slices for 4 hr only and [3-^{14}C]coumarin metabolism to total polar products (A) and metabolite(s) that bound covalently to tissue slice proteins (B) determined. Values are means ± SEM of four experiments.

in lung slices were unknown metabolites and products of the 3-hydroxylation pathway, with only small amounts of 7-HC being formed (data not shown). [3-^{14}C]Coumarin metabolism to 7-HC in rat liver and lung slices after 4 hr was 38 ± 19 (mean ± SEM of four experiments) and 55 ± 33 pmol/mg slice protein, respectively, which was only 1.3 and 2.3%, respectively, of [3-^{14}C]coumarin metabolism to total polar products.

Discussion

Many studies have demonstrated that precision-cut liver slices are a valuable *in vitro* model system for studying the metabolism and toxicity of xenobiotics (Azri *et al.*, 1990; Brendel *et al.*, 1987; Sipes *et al.*, 1987). Precision-cut liver slices from various species have been shown to be able to metabolize a wide range of xenobiotics including chlorobenzenes, cyclosporin A, diazepam 2,6-dinitrotoluene, lidocaine, paracetamol and tolbutamide (Barr *et al.*, 1991a,b; Chapman *et al.*, 1993; Dogterom, 1993; Miller *et al.*, 1993; Olinga *et al.*, 1993; Vickers *et al.*, 1992). Although precision-cut tissue slices from extrahepatic tissues have been used for studies of xenobiotic-induced toxicity (Fisher *et al.*, 1994; Parrish *et al.*, 1992; Ruegg *et al.*, 1989; Schwade and Thompson, 1994), they do not appear to have been extensively used for studies of xenobiotic metabolism. In one investigation Vickers *et al.* (1992) examined the metabolism of cyclosporin A by human liver, kidney and intestinal slices. Cyclosporin A was metabolized by all three tissue slice preparations and the data demonstrated that the intestinal metabolism of cyclosporin A contributes to the first-pass effect of this drug.

With respect to the lung, the present data demonstrate that precision-cut slices from this organ can metabolize 7-ethoxycoumarin and coumarin to various products. Studies with the rat and other species have demonstrated that the lung contains a range of xenobiotic metabolizing enzymes, including cytochrome *P*-450-dependent mixed function oxidase activities, epoxide hydrolase, glutathione *S*-transferase and UDPglucuronosyltransferase (Benford and Bridges, 1986; Lake *et al.*, 1979; Vainio and Hietanen, 1980). Xenobiotic metabolizing enzymes are not uniformly distributed throughout the various lung cell types (Benford and Bridges, 1986; Devereux and Fouts, 1981; Devereux *et al.*, 1981): for example, the Clara cell contains high levels of cytochrome *P*-450 isoenzymes, which accounts for the susceptibility of this cell type to certain lung toxins such as 4-ipomeanol (Boyd, 1977; Devereux *et al.*, 1981).

In the present study 7-ethoxycoumarin was metabolized by rat liver and lung slices to 7-HC, which was extensively conjugated with sulfate. On a unit of protein basis, total 7-ethoxycoumarin metabolism in rat lung slices after 4 hr was about 47% of that in rat liver slices. The greater metabolism of 7-ethoxycoumarin by liver than lung slices is in agreement with previous *in vitro* studies where *O*-deethylase activity has been measured in liver and lung preparations (Lake *et al.*, 1979; Vainio and Hietanen, 1980). [3-^{14}C]Coumarin was metabolized by rat liver and lung slices to various polar products and to metabolite(s) that bound covalently to proteins. Whereas rates of [3-^{14}C]coumarin metabolism to total polar products in rat liver and lung slices were fairly similar, covalent binding in lung slices after 4 hr was only 35% of the rate in liver slices. Previous studies with rat liver microsomes have demonstrated that coumarin is metabolized to product(s) that bind covalently to proteins, although the role, if any, of covalent binding in coumarin-induced hepatotoxicity requires further elucidation (Lake, 1984; Lake *et al.*, 1992). As would be expected from previous studies with hepatic microsomes and in the intact animal (Cohen, 1979; Fentem and Fry, 1992; Lake *et al.*, 1992), 7-HC was only a very minor metabolite of coumarin in both rat liver and lung slices.

These results demonstrate that the technique of precision-cut tissue slices may be used to provide information on the hepatic and extrahepatic phase I and phase II metabolism of xenobiotics. Indeed, tissue slices appear to have several advantages over other *in vitro* systems: for example, compared with subcellular fractions (e.g. microsomes) all phase I and II xenobiotic metabolizing enzymes are present in tissue slices. Although integrated hepatic metabolism can be studied *in vitro* using hepatocytes, tissue slices are more representative of the whole liver as all the cell types are present. By employing suitable agarose instilling or embedding techniques, it should be possible to prepare slices from a wide range of extrahepatic tissues from both experimental animals and humans. Precision-cut tissue slices thus have potential as a rapid and valuable *in vitro* model system for comparing hepatic and extrahepatic xenobiotic metabolism. Moreover, such systems may be used to investigate species differences in the metabolism of drugs, environmental chemicals and other xenobiotics.

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