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THE ISOLATION OF RAT LUNG CELLS FOR THE PURPOSE OF STUDYING DRUG METABOLISM

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Abstract—A method was developed for the isolation of viable cells from rat lungs. The cells were a heterogeneous population, which maintained a high percentage viability for up to 3 hr on incubation at 37°. Reduced cofactor levels (NADH, NADPH) decreased on incubation, but ATP remained constant. The cells were active in the metabolism of the two xenobiotics studied. A model compound, 7-ethoxycoumarin, was *O*-deethylated and subsequently conjugated with glucuronic acid and sulphate, whilst a pharmaceutical agent, *N*-acetylcysteine, was de-acetylated.

Xenobiotics, both toxic and therapeutic in nature, can reach the lung cells via the inhaled air or via the blood. It is now very well documented that the lungs are capable of metabolizing such compounds [1–4], with the production of metabolites which are less or more active than the parent compound. The investigation of such metabolism will help to elucidate the susceptibility of the lung cells to toxic agents, and, in the case of therapeutic drugs, will indicate which compound is most likely to be the active agent.

There are a number of different systems which can be used to study pulmonary xenobiotic metabolism [5]. Subcellular fractions have been used quite extensively in the past [1–3], but these usually require the addition of cofactors, often at unphysiological concentrations, and are usually used when studying individual reactions. Tissue explants have also been used for such studies [6] but the viability of such a system is difficult to determine, there are likely to be artefactual barriers to the diffusion of substrates and metabolites, and the results can be difficult to quantitate precisely. The use of the isolated, perfused lung can overcome some of these problems, but it is a difficult system to work with, requires specialized equipment and can be kept viable for a maximum period of only 4 hr, the usual time period used being much shorter [4, 5, 7–9].

Isolated cells have already proved to be an invaluable tool in the study of drug metabolism by the liver, kidney and small intestine [10–15], but so far there have been very few reports of the successful use of isolated lung cells for such studies. Such reports as do exist have been mainly restricted to the use of rabbit lung cells [16–18], with one report in which cultured rat lung cells were used [19]. The rabbit is not a commonly used animal in toxicological studies, and therefore development of a method for isolating cells from the rat lung for the purpose of studying xenobiotic metabolism was the goal of the presently reported endeavour.

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MATERIALS AND METHODS

Chemicals. Protease type VII; bovine serum albumin, fraction V; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and trypan blue were purchased from the Sigma Chemical Co. (St. Louis, MO). 7-Ethoxycoumarin was synthesized according to the method of Ullrich and Weber [20]. Lumagel scintillation fluid was purchased from Lumac B.V. (Schaesberg, The Netherlands). ^{14}C-Labelled *N*-acetylcysteine (7 mCi/mmole), labelled in the cysteine moiety, was synthesized by Inveresk Research International (Edinburgh, U.K.). Bondapac NH_2 HPLC columns were purchased from Waters Associates A.B. (Partille, Sweden). All other chemicals and solvents used were of analytical or reagent grade, purchased through local chemical suppliers.

Animals. Male Sprague–Dawley rats (180–200 g), allowed free access to food and water, were used throughout.

Cell isolation. The rat was anaesthetized with ether. The abdominal cavity was opened, and heparin (0.2 ml, 5000 IE/ml) was injected into the caval vein. The thoracic cavity was then opened, the thymus removed, and the blood perfused out of the lungs by insertion of a cannula (size 12) into the pulmonary artery, via the right ventricle, the left auricle being cut to allow the perfusate to escape. The perfusion medium (150 ml of Krebs buffer containing 2.5% bovine serum albumin and 5 mM glucose) was maintained at 37° and bubbled with carbogen (95% O_2, 5% CO_2) throughout. During the perfusion, the lungs, heart and part of the trachea (approximately 1.5 cm above the tracheal junction) were removed together, and perfusion was continued *in vitro* with a recirculating system. When perfusion was complete, the lungs appeared white, and the cannula was then removed. The heart and blood vessels were dissected away and a cannula, through which the enzyme solution (150 ml of 1 mg/ml protease type VII, 1 mM EDTA in Ca^{2+}- and Mg^{2+}-free buffer, pH 7.4, 37°) was perfused, was inserted into

the trachea. Perfusion, which was again recirculating, was continued for approximately 15 min, depending on the age of the rat, after which time the lungs were very soft to the touch, and the cells could be readily dispersed into Krebs–HEPES buffer, containing 5 mM glucose (pH 7.4). The cell suspension was filtered through nylon bolting cloth (100-μm pore size) and the cells harvested by centrifugation (80 g for 5 min). The cells were washed once, and finally resuspended in the same buffer, and used at a concentration of 4×10^6 cells/ml.

Assays. Cell viability was determined by the trypan blue exclusion method, and by the ability of the cells to exclude NADH [21].

Protein content was determined, after cell lysis, by the method of Lowry *et al.* [22].

Reduced and oxidized nicotinamide adenine dinucleotides were determined as in Ref. 23, using 1 ml of cell incubate.

ATP was measured, using 1 ml of cell incubate, as described in Ref. 24.

Reduced and oxidized glutathione were determined by the HPLC method of Reed and Beatty [25].

Metabolites of *N*-acetylcysteine were determined using the same HPLC technique as for glutathione [25], employing ^{14}C-labelled *N*-acetylcysteine in order to quantitate the metabolites eluted from the HPLC column.

Metabolites of 7-ethoxycoumarin were determined in 2-ml aliquots of cell suspension using the method detailed in Ref. 14. The substrate concentrations used were 100 and 200 μM, with no additional cofactors.

Cell identification. Type II epithelial cells were identified using phosphine 3R [16]. Clara cells were identified as described by Devereux and Fouts [26]. Ciliated cells were easily identified under a light microscope from their characteristic appearance.

RESULTS

The presently reported method for isolation of rat lung cells was adapted from that used by Devereux and Fouts to isolate cells from the rabbit lung [17]. In the development of this method, various ways of administering the enzyme solution to the lungs, and different lytic enzymes were tried. Instillation of the enzyme solution into the lungs via the trachea, a method previously used by Devereux and Fouts with the rabbit lung [16, 17], proved to be ineffective with the rat lung, due to continuous leakage of the solution from the lung, presumably via the vascular system. Administration of the enzyme solution via the pulmonary artery was also less efficient as regards tissue digestion than the finally adopted method of continuous perfusion via the trachea. The enzyme solutions tested in this respect were: Sigma protease type I (1 mg/ml) with 1 mM EGTA or 1 mM EDTA, trypsin (0.5 mg/ml), collagenase (1 mg/ml) in a Ca^{2+}- and Mg^{2+}-containing buffer, collagenase (1 mg/ml) plus elastase (0.1 mg/ml), Sigma protease type VII with 1 mM EGTA or 1 mM EDTA. Trypsin and protease type I did not digest the lung tissue even with a 30-min incubation. Collagenase was found to be toxic to lung cells and the few cells (40×10^6) obtained using collagenase were less than 50% viable. Protease type VII wtih EGTA proved to be less efficient in digesting the lung tissue than it was with EDTA. Thus the procedure finally adopted was, as described in Materials and Methods, perfusion, via the trachea, of a solution containing protease type VII (1 mg/ml) and EDTA (1 mM) for approximately 15 min.

Table 1. Properties of the isolated lung cell preparation

Cell yield per rat (million)	160 ± 40	(N = 38)
Viability (per cent)	84 ± 5	(N = 38)
Protein (mg/10^6 cells)	0.192 ± 0.048	(N = 7)
Glutathione (nmoles/10^6 cells)	0.16 ± 0.13	(N = 13)
GSSG (nmoles/10^6 cells)	0.02 ± 0.03	(N = 13)
De-acetylation of *N*-acetylcysteine (pmoles/10^6 cells/min)	47.7 ± 1.0	(N = 4)

Values are expressed as the means ± S.D., with the number of separate determinations given in parentheses. *N*-Acetylcysteine (1 mM) was incubated at 37° for up to 1 hr with 4×10^6 lung cells/ml and metabolites determined as described in Materials and Methods.

As shown in Table 1, the yield of lung cells obtained by this newly-developed method was within the range 1.2×10^8–2×10^8 cells from one rat. Viability of the cells, as assessed by their ability to exclude trypan blue, was usually over 80% immediately after isolation and, as shown in Fig. 1, remained high for up to 3 hr on incubation at 37°. When viewed under the light microscope, the cells were of a heterogeneous appearance, and of various sizes. The cell suspension contained approximately 15% type II epithelial cells, 1.14% ciliated epithelial cells and less than 1% Clara cells. Other cell types which are present, but have not been quantitated, include macrophages, mucus-producing cells (confirmed by the production of mucus during cell incubations),

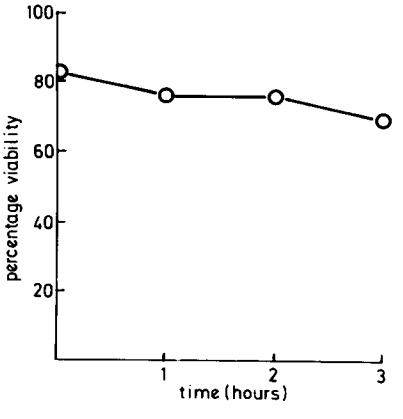

Fig. 1. The viability of isolated lung cells, as assessed by their ability to exclude NADH, on incubation at 37° in Krebs–HEPES buffer containing 5 mM glucose and an amino acid mixture [40] (pH 7.4). Results represent the means of three separate determinations.

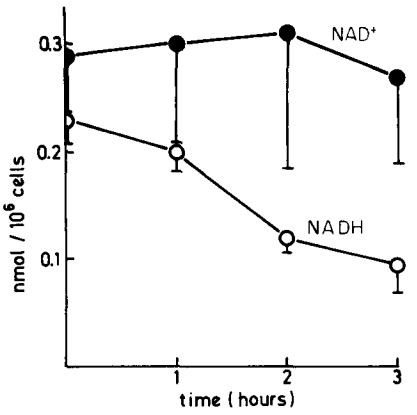

Fig. 2. Levels of nicotinamide adenine dinucleotides in isolated lung cells, on incubation at 37° in a Krebs–HEPES buffer containing 5 mM glucose and an amino acid mixture [40] (pH 7.4). Results represent the means of three separate determinations. Bars depict S.E.M.

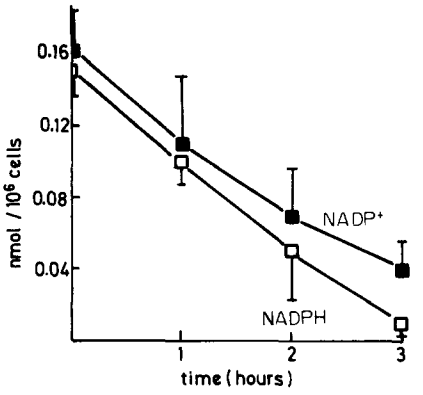

Fig. 3. Nicotinamide adenine dinucleotide phosphate levels in isolated lung cells on incubation at 37° in a Krebs–HEPES buffer containing an amino acid mixture [40] (pH 7.4). Results represent the means of three separate determinations. Bars depict S.E.M.

lymphocytes and occasionally red blood cells. Overall the cells were about one-fifth of the size of liver cells, which is reflected by their protein content (Table 1) of less than 0.2 mg per 10^6 cells.

Reduced glutathione was very low in these cells, and decreased on incubation at 37°; consequently there was a large variation in the results obtained (Table 1). Glutathione disulfide was usually absent from the lung cells, and was detected in only three of the 13 cases (Table 1).

The cells were active in deacetylating *N*-acetylcysteine (Table 1), with the products formed being cysteine (65.5%) and *N*-acetylcystine (34.5%).

The level of NADH in these lung cells decreased, over 3 hr at 37°, to half of the initial value, whilst NAD^+ increased slightly during the first 2 hr, and then decreased during the third hour (Fig. 2). However, both the NADPH and $NADP^+$ concentrations decreased on incubation at 37° (Fig. 3). The level fell less than 20% during the first hour of incubation, and thereafter remained fairly constant (Fig. 4).

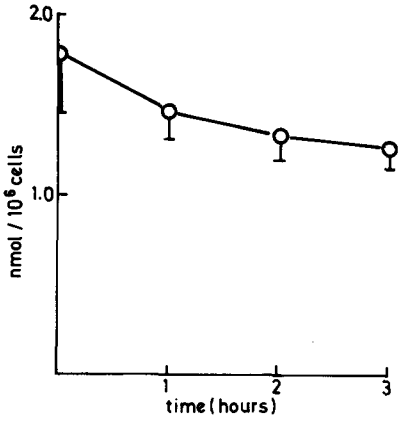

Fig. 4. ATP level in isolated lung cells on incubation at 37° in a Krebs–HEPES buffer containing 5 mM glucose and an amino acid mixture [40] (pH 7.4). Results represent the means of five separate determinations. Bars depict S.E.M.

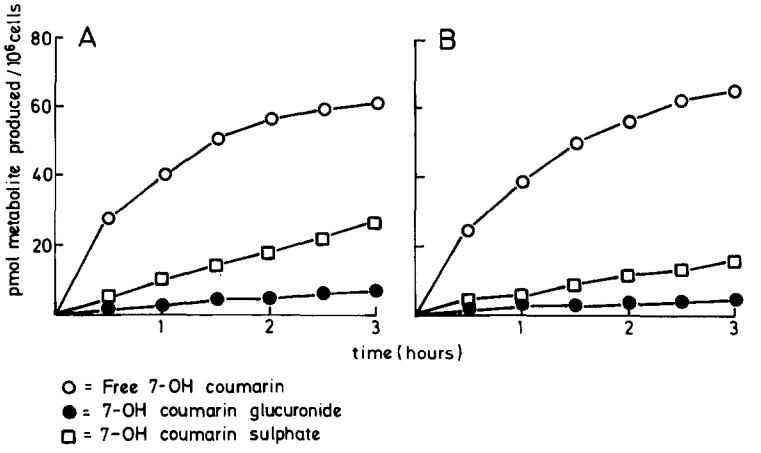

Fig. 5. Metabolism of 7-ethoxycoumarin by isolated lung cells. Substrate concentrations used were 100 μM (A) and 200 μM (B), and incubations were performed in rotating, round-bottomed flasks at 37°, under an air atmosphere. The medium used was a Krebs–HEPES buffer containing glucose (5 mM) and an amino acid mixture [40] (pH 7.4). Results represent the means of three separate determinations.

The metabolism of 7-ethoxycoumarin by the lung cells was studied using two different substrate concentrations, 100 and 200 μM. The higher substrate concentration resulted in 10% fewer products being formed at each time point, this being manifested as fewer conjugates being present (Fig. 5A and B).

DISCUSSION

Previous attempts to isolate cells from rat lungs have, in general, met with little success, resulting in low yields of cells or poor viability [27–32]. One group [33], who compared the effectiveness of different enzymes in the dispersal of lung tissue, was more successful, obtaining 1.5×10^8 cells from one animal, but they reported only a morphological characterization of their preparation.

The presently reported method for the isolation of rat lung cells yielded between 1.2×10^8 and 2.0×10^8 cells from one animal. Yields of 2.5×10^8 have been obtained occasionally, which indicates that previous estimates of the total lung cells available for dispersal have been somewhat low [28, 33]. The viability of the lung cell preparation was always close to 80% (Table 1) and a high percentage of viable cells was maintained for up to 3 hr (Fig. 1).

There appears to be no literature available on the exact proportions of the different cell types found in the lung *in vivo*, but there are over 40 different cell types found in the lung [34], and no one cell type predominates. The presently reported method for isolation of rat lung cells was an attempt to obtain as many cells as possible from one pair of lungs in the hope that the cell population would be representative of the lung as a whole. It is not the purpose of the present communication to report a complete morphological characterization of the lung cell population obtained, since such characterization will be presented in a separate publication. This communication reports some biochemical properties of these cells, especially in relation to drug metabolism.

The cellular content of NADH and NADPH fell on incubation at 37° for 3 hr. At the same time there was a slight rise in the NAD^+ level, followed by a small decrease over the third hour, but the $NADP^+$ level of these cells fell almost in parallel with the decrease in NADPH (Figs 2 and 3). Thus, these cells appear to be unable to maintain the intracellular level of reduced cofactors under the incubation conditions used. At present the reason for this is not clear, though it appears, to be linked to the intracellular reduced glutathione level, which also decreases with incubation time (unpublished observations). Further investigations into the energy requirements and glutathione turnover of these cells are at present being performed. The ATP level of the lung cells is, however, maintained rather well (Fig. 4), which indicates that mitochondrial function is unimpaired.

Drug metabolism by these lung cells was studied using one model substrate, 7-ethoxycoumarin (Fig. 5) and a therapeutically used agent, *N*-acetylcysteine (Table 1). 7-Ethoxycoumarin is initially *O*-deethylated by a cytochrome P-450-dependent pathway, and the 7-ethoxycoumarin produced is then usually conjugated with glucuronic acid and sulphate [14, 35], and can thus be used to investigate the relationship between these two phases of drug metabolism. One group has previously reported *O*-deethylation of 7-ethoxycoumarin by lung cells isolated from the rabbit [16, 18]. The values reported have varied from 20.2 to over 100 pmoles metabolized/mg protein/min, in the presence of 1 mM NADPH. In the present investigation the rate of *O*-deethylation of 7-ethoxycoumarin over the first 30 min of incubation was 57 pmoles/mg protein/min with no added cofactors present, The rate of metabolism decreased with time (Fig. 5), which is possibly a reflection of the decrease in the NADPH level of these cells (Fig. 3) (since NADPH is required for the cytochrome P-450 enzyme system). However, the rate of conjugation of the 7-hydroxycoumarin released did not decrease with time (Fig. 5). However, it appeared that 100 μM was a saturating substrate concentration with these cells (Fig. 5A). With 200 μM 7-ethoxycoumarin there were fewer sulphate and glucuronide conjugates produced (Fig. 5B) and a lower amount of total metabolites with no adverse effect on cell viability. Although a larger range of substrate concentrations needs to be studied before any definite conclusions can be reached as to the optimum substrate concentration, in preliminary experiments 50 μM 7-ethoxycoumarin resulted in fewer metabolites being formed than with 100 μM 7-ethoxycoumarin, so indications are that the optimal concentration of 7-ethoxycoumarin will be close to 100 μM.

The isolated lung cells were, on the whole, not very efficient at conjugating the 7-hydroxycoumarin released, which is in marked contrast to cells from the small intestine, kidney and liver [12, 14, 35], where the majority of the metabolites formed from 7-ethoxycoumarin are conjugates. At present it is not known if this is due to the levels of the sulfate and glucuronyl transferases, their affinities or the production of cofactors, which could be limiting. However, the lung cell preparation does produce quantities of mucus, the synthesis of which requires PAPS, though this could be taking place in cell types different from those responsible for xenobiotic metabolism. If it is generally true that the lung is poor at conjugating, it could be one of the reasons why the lung is more susceptible to carcinogens than is the liver or small intestines.

N-Acetylcysteine is a pharmaceutical compound which is used as an antidote to paracetamol poisoning [36] and as a mucolytic in cases of chronic bronchitis [37]. At present it is unclear what is the active agent in the lung, though in the liver it is thought to act as a glutathione precursor [10, 38]. In the present investigation, the lung cells deacylated *N*-acetylcysteine at a rate of 48 pmoles per 10^6 cells in 1 min, when the substrate concentration used was 1 mM (Table 1). This substrate concentration is probably considerably higher than that likely to be approached *in vivo* in the lung, so this rate of metabolism means that cysteine, or a further metabolite of cysteine (e.g. glutathione), could be the active agent, rather than *N*-acetylcysteine itself. These results also confirm that the lung has an active esterase, a fact which has been previously reported for an isolated, perfused lung preparation [39].

The presently reported method for the isolation of cells from the rat lung results in a high yield of viable cells, which are active in the metabolism of xenobiotics, and which could be used in toxicological investigations. Further studies are at present being performed using these cells to investigate the effects of toxic compounds on the lung, and to study the pulmonary turnover of glutathione. However, it is already apparent that this cell system will prove to be a valuable tool in the investigation of xenobiotic metabolism, the effect of toxic agents, and the mode of action of pharmaceutical compounds in the lung.

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