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INDUCTION OF ALKOXYRESORUFIN *O*-DEALKYLASES AND UDP-GLUCURONOSYL TRANSFERASE BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE IN PRIMARY CULTURES OF PORCINE CILIARY EPITHELIAL CELLS

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Abstract—Our previous studies have shown that drug-metabolizing activities in the eye are highest in the ciliary body, a tissue responsible for aqueous humor production. In this work, we have separated nonpigmented epithelial (NPE) cells and pigmented epithelial (PE) cells from porcine ciliary body and determined basal and induced activities of 7-ethoxyresorufin (ER) *O*-dealkylase, 7-pentoxyresorufin (PR) *O*-dealkylase, and UDP-glucuronosyl transferase (UDP-GT) using primary cultures of separated cells. ER and PR activities were associated primarily with NPE cells and were very low in PE cells. Treatment of NPE cells with phenobarbital (PB) for 48 hr resulted in about a 4-fold increase in PR *O*-dealkylase activity but only a 1.3-fold rise in ER *O*-dealkylase activity. Conversely, 3-methylcholanthrene (MC) treatment augumented the ER *O*-dealkylase activity of NPE cells 6 times over the basal activity in 48 hr but had little effect on PR *O*-dealkylase activity. Both NPE and PE cells had low basal UDP-GT activities. UDP-GT activity increased about 5-fold in PB-treated PE cells and about 4-fold in PB-treated NPE cells in 48 hr. The results of MC treatment were similar to those of PB treatment; enhancement of UDP-GT was more pronounced in PE cells than in NPE cells. Induction by PB and MC of ER *O*-dealkylase, PR *O*-dealkylase and UDP-GT activities in ciliary NPE and PE cells was inhibited almost completely by 3.5 μM cyclohexamide and 40 nM actinomycin D. The heterogeneous distribution of these enzymes suggests that a harmonious interplay between NPE and PE cells is important for metabolic detoxification of blood plasma prior to aqueous humor formation.

The liver is the major organ for drug metabolism in the body. Studies in recent years, however, have demonstrated that virtually all organs, including the eye, possess drug-metabolizing capability [1]. Our previous studies and studies from other laboratories have shown that, in the eye, drug-metabolizing activities are highest in the uveal tissues, especially in ciliary epithelial processes [2–4]. The ciliary epithelium is composed of two cell layers, i.e. pigmented and nonpigmented cell layers. One of the important functions of the ciliary epithelium is secretion of the aqueous humor into the anterior chamber, a process involving both pigmented and nonpigmented cells for transport of water, ions, and nonproteinous components of blood plasma. Since the aqueous humor provides nutrients to transparent tissues such as the lens and cornea, it is essential to ensure that the secreted fluid does not contain substances harmful to the transparent tissues. Toxicity cataracts such as those induced by naphthalene, dinitrophenol, and corticosteroids are believed to be caused by a disturbance of lenticular metabolism by these chemicals or their metabolites secreted in the aqueous humor [5]. In spite of their clinical importance, very little is known about the properties of enzymes involved in drug metabolism and detoxification in the ciliary epithelium. In this work, therefore, we have investigated the distribution and inducibility of Phase I enzymes (phenobarbital-inducible 7-pentoxyresorufin *O*-dealkylase and 3-methylcholanthrene-inducible 7-ethoxyresorufin *O*-dealkylase [6]) and Phase II enzyme (UDP-glucuronosyl transferase†) in primary cultures of nonpigmented and pigmented cells separated individually from porcine ciliary processes. The physiological significance of the results is discussed in regard to the cellular defense mechanisms of the ciliary epithelial cells against adverse effects of xenobiotic compounds.

MATERIALS AND METHODS

Separation of ciliary epithelial cells from porcine eye. Porcine eyes were collected at a local abattoir and brought to the laboratory on ice within an hour after slaughtering of animals. The eyes were washed with deionized water, immersed in 70% isopropylalcohol for 10 sec, washed with sterile saline containing gentamycin (50 μg/ml) (GIBCO Co., Grand Island, NY) and cut along the equator posterior to the lens to collect the anterior cup. After removal of vitreous body, lens, and retina, the anterior cup was cut in halves and incubated for

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† Abbreviations: PE, pigmented epithelial cells; NPE, nonpigmented epithelial cells; ER, 7-ethoxyresorufin; PR, 7-pentoxyresorufin; UDP-GT, UDP-glucuronosyl transferase; PB, phenobarbital; and MC, 3-methylcholanthrene.

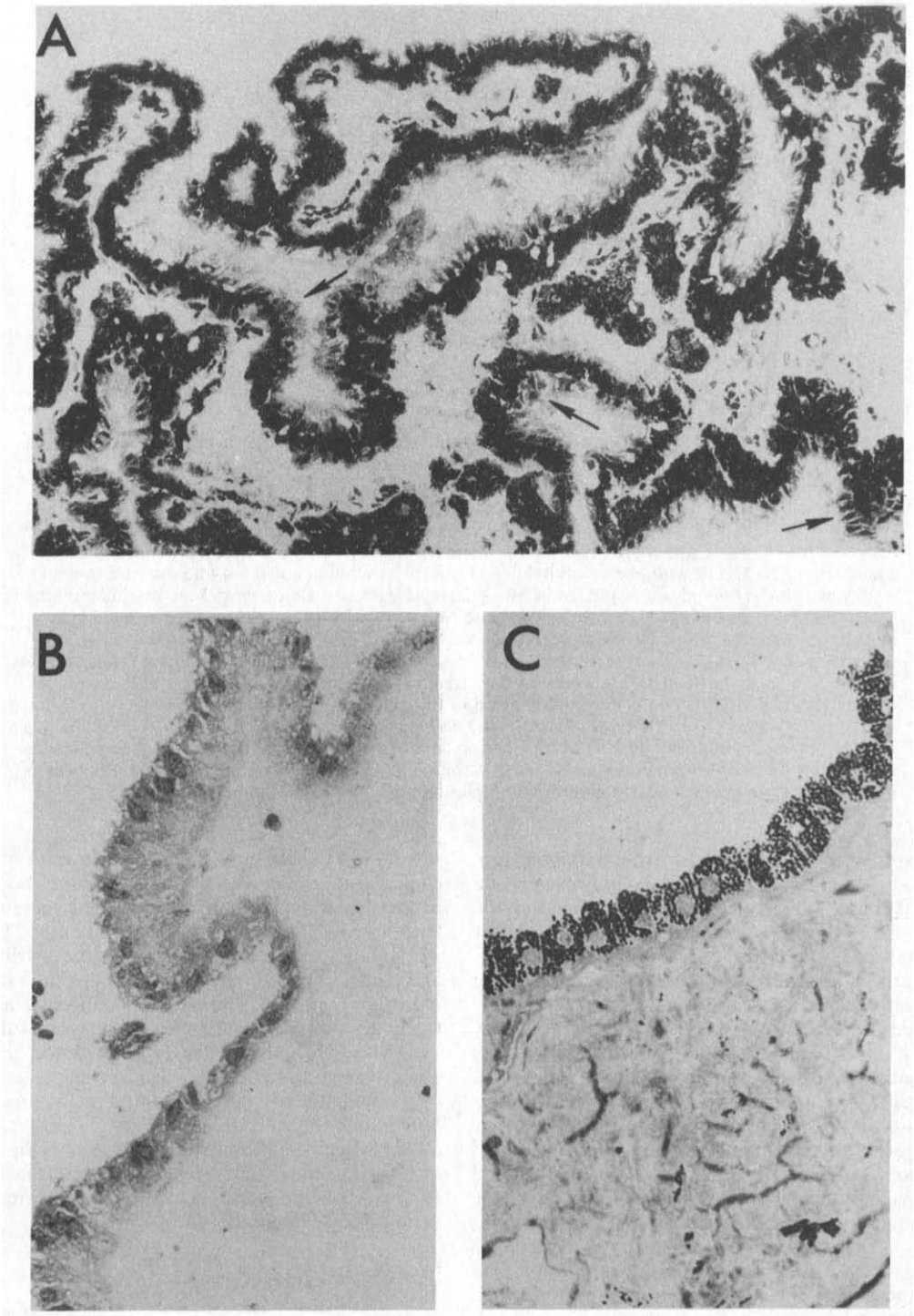

Fig. 1. Light micrographs of porcine ciliary epithelial cells. (A) Ciliary processes composed of NPE cells (arrows), PE cells and stroma. (B) Sheet of NPE cells separated from PE cells. (C) PE cells still attached to the stroma after separation of NPE cells. Original magnification: 38× for A and 150× for B and C.

5–7 min in 0.02% EDTA in phosphate-buffered saline (the Sigma Chemical Co., St. Louis, MO) to loosen the cellular contact between the pigmented epithelial (PE) cells and the nonpigmented epithelial (NPE) cells. NPE cells, together with remaining vitreous, were detached carefully from PE cells with a pair of tweezers. PE cells were then separated from the stroma by gentle suction into a pipette.

NPE cells attached to the vitreous were obtained as sheets, while PE cells were dispersed. Isolated NPE contained 7% PE and PE contained 0.5% NPE and 6.5% other cells (lymphocytes, red blood cells, etc.). NPE cells and PE cells were collected individually in Dulbecco's Modified Eagle Medium (GIBCO) containing 17% fetal bovine serum (GIBCO) and garamycin (50 μg/mL) (Schering Co., Kenilworth, NJ) and incubated at 37° in CO_2/air (5%/95%) for a given length of time, i.e. 24, 48, 72, or 96 hr in the presence or absence of inducer compound. Each culture dish contained 5×10^6 cells in 25 mL medium. The final concentrations of inducers added to the medium were 2 mM PB and 1 μM MC. Phenobarbital (PB), 3-methylcholanthrene (MC), cyclohexamide and actinomycin D were purchased from Sigma.

Preparation of microsomal fraction. Cultured NPE and PE cells were harvested and homogenized immediately for 2 min with a Tissumizer (Tekmar, Cincinnati, OH) in 0.1 M Tris–0.25 M sucrose (pH 7.5) containing protease inhibitors [10 μg/mL of leupeptin, 5 mM EDTA and 1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) (all from Sigma)] and centrifuged at 10,000 *g* for 20 min. The supernatant was collected and centrifuged at 100,000 *g* for 60 min. The microsomal pellet thus obtained was washed and suspended in 0.05 M Tris–0.25 M sucrose (pH 7.5), and used immediately for enzyme assay.

Enzyme assays. 7-Pentoxyresorufin (PR) *O*-dealkylase and 7-ethoxyresorufin (ER) *O*-dealkylase activities were determined according to Lubet *et al.* [7]. To a mixture of 400 μL of 50 mM Tris buffer (pH 7.5) containing $MgCl_2$ (10 μmol), 5 μL of 1 mM PR or 2 μL of 0.5 mM ER, and 2–20 μL of microsomal suspension (approx. 0.5 mg protein/mL), 130 μL of 0.5 mM NADPH was added and a fluorescence increase at 586 nm (with excitation light at 522 nm) was followed using a Shimadzu fluorospectrometer RF-540 (Giangarlo Scientific Co., Pittsburgh, PA). To investigate the effect of anti-cytochrome $P450_{PB}$ antibodies and anti-cytochrome $P450_{MC}$ antibodies on PR *O*-dealkylase and ER *O*-dealkylase activities, guinea pig serum containing these antibodies was diluted to desired concentrations with 0.1 M Tris–0.25 M sucrose before the antibodies were added to the reaction mixture for preincubation for 30 min at 20°. The antibodies raised against cytochrome $P450_{PB}$ and cytochrome $P450_{MC}$ purified from rabbit liver were used previously for immunoprecipitation of the individual cytochrome P450 isozymes of mutant rats [8]. Protein was determined with bicinchoninic acid (Pierce Chemical Co., Rockford, IL) according to Smith *et al.* [9].

UDP-glucuronosyl transferase (UDP-GT) activity was assayed by measuring the transfer of glucuronic acid from UDP-glucuronate to *p*-nitrophenol. A procedure described by Mulder and Van Doorn [10] has been modified. To a reaction mixture (0.45 mL, pH 7.3) containing Tris–HCl (2.6 μmol), $MgCl_2$ (0.17 μmol), UDP-glucuronic acid (0.11 μmol), glucuro-1,4-lactone (0.07 μmol), and *p*-nitrophenol (0.02 μmol), 0.1 mL (about 10 μg protein) of microsomal suspension (containing 0.5% Triton X-100 to activate enzyme) was added. After incubation for a given time (30–120 min at 37°), the mixture

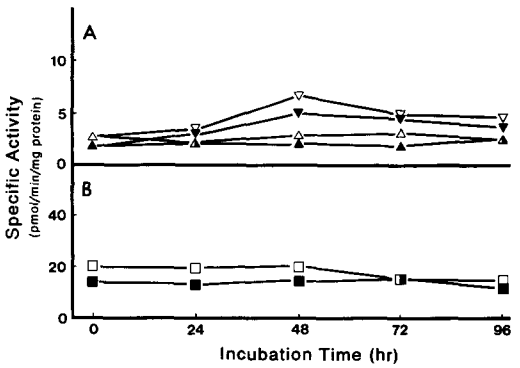

Fig. 2. Basal activities of PR and ER *O*-dealkylases in PE and NPE cells and induced activities of PR and ER *O*-dealkylases in PE cells. (A) Basal and induced levels of PR and ER *O*-dealkylase in PE cells. Key: (▲) PR *O*-dealkylase; (△) ER *O*-dealkylase; (▼) PR *O*-dealkylase after PB (2 mM) treatment; and (▽) ER *O*-dealkylase after MC (1 μM) treatment. (B) Basal PR and ER *O*-dealkylase activities of NPE cells. Key: (■) PR *O*-dealkylase; and (□) ER *O*-dealkylase. Standard deviation values were within ± 20% of the mean values; N = 3.

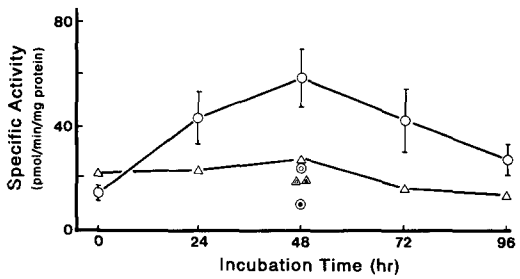

Fig. 3. Induced levels of PR and ER *O*-dealkylase activities of PB-treated NPE cells. Key: (○) PR *O*-dealkylase; (△) ER *O*-dealkylase; (◉) and (◎) inhibition of PR *O*-dealkylase induction by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively; and (▲) and (▲) inhibition of ER *O*-dealkylase induction by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively. Bars indicate standard deviations (N = 4).

was mixed with 1 mL of trichloroacetic acid and centrifuged for 10 min at 10,000 *g* to collect the supernatant. One milliliter of the supernatant was mixed with 30 μL of saturated NaOH to pH 12 and the absorbance at 405 nm was determined. For a blank, a reaction mixture containing all ingredients except UDP-glucuronic acid was treated in a similar manner.

RESULTS

Ciliary epithelial cells. Figure 1 shows the two-layer architecture of porcine ciliary epithelium (A), isolated NPE cells (B), and PE cells before separation from the stroma (C). The viability of both NPE and PE cells, as determined by uptake of trypan blue, was 90–98% for all intervals of incubation in

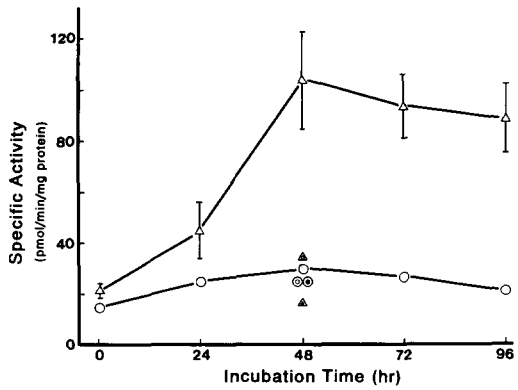

Fig. 4. Induced levels of PR and ER *O*-dealkylase activities of MC-treated NPE cells. Key: (○) PR *O*-dealkylase; (△) ER *O*-dealkylase; (⊙) and (◎) inhibition of PR *O*-dealkylase induction by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively; and (▲) and (▲) inhibition of ER *O*-dealkylase induction by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively. Bars indicate standard deviations (N = 4).

the presence of inducers. Hence, inducers at concentrations used were not cytotoxic.

PR O-dealkylase and ER O-dealkylase activities. Basal (i.e. untreated) levels of PR *O*-dealkylase and ER *O*-dealkylase activities of PE cells and NPE cells are shown in Fig. 2A and Fig. 2B respectively. The two *O*-dealkylase activities of NPE cells were about 10 times higher than those of PE cells. These activities remained essentially unchanged during culture of both NPE and PE cells over a period of 96 hr. The PR *O*-dealkylase activity of PE cells incubated with 2 mM PB for 48 hr was 1.8-fold higher than the cells similarly incubated without PB. Incubation of PE cells with 1 μM MC for 48 hr resulted in about a 2.5-fold increase in ER *O*-dealkylase activity. These *O*-dealkylase activities of PE cells, whether basal or induced, were considerably lower than the comparable activities of NPE cells (see below) and were almost insignificant.

Treatment of NPE cells with PB for 48 hr enhanced PR *O*-dealkylase activity 3.9 times (Fig. 3) over the basal level of PR *O*-dealkylase activity (Fig. 2B). In contrast, the ER *O*-dealkylase activity of PB-treated NPE cells increased only 1.3-fold. The induction by PB of PR *O*-dealkylase activity in NPE cells was inhibited completely by 3.5 μM cyclohexamide or 40 nM actinomycin D (Fig. 3). The ER *O*-dealkylase activity of NPE cells was augmented about 6 times by treatment with MC for 48 hr (Fig. 4), while the inducer compound had little effect on the PR *O*-dealkylase activity of NPE cells. The MC-dependent induction of ER *O*-dealkylase activity was abolished completely by cyclohexamide or actinomycin D (Fig. 4). The apparent K_m values for ER and PR of *O*-dealkylases of NPE cells were similar; ER: 1.1 μM (basal) and 3.0 μM (MC-induced) and PR: 7.4 μM (basal) and 8.2 μM (PB-induced). As shown in Table 1, anti-cytochrome $P450_{PB}$ antibodies inhibited the PR *O*-dealkylase activity, but not the ER *O*-dealkylase activity, of PB-treated NPE cells. Conversely, anti-cytochrome $P450_{MC}$ antibodies inhibited the ER *O*-dealkylase activity of MC-treated NPE cells but had little effect on the PR *O*-dealkylase activity of MC-treated cells.

Induction by PB of UDP-GT activity in the microsomal fractions of NPE and PE is presented in Fig. 5. Both NPE and PE cells had low basal UDP-GT activities which did not change during incubation over a period of 96 hr. The transferase activity of PB-treated PE cells was increased 4.7 times over the basal activity in 48 hr, while the increase of transferase activity of PB-treated NPE

Table 1. Inhibition of PR *O*-dealkylase and ER *O*-dealkylase activities by antibodies against cytochrome $P450_{PB}$ and cytochrome $P450_{MC}$

	PR *O*-dealkylase activity (pmol/min/mg)	Inhibition (%)
PB-treated microsomes		
Without antibodies	77.1	0
With anti-cytochrome $P450_{PB}$ antibodies (900× dil.)	44.9	42
With anti-cytochrome $P450_{MC}$ antibodies (450× dil.)	77.8	0
	ER *O*-dealkylase activity (pmol/min/mg)	**Inhibition (%)**
MC-treated microsomes		
Without antibodies	26.4	0
With anti-cytochrome $P450_{MC}$ antibodies (450× dil.)	7.5	72
With anti-cytochrome $P450_{PB}$ antibodies (900× dil.)	22.7	0

Values are the means of three determinations. The microsomes were prepared from NPE cells incubated with either 2 mM PB or 1 μM MC for 48 hr.

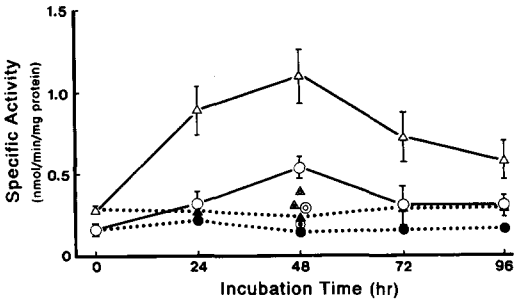

Fig. 5. Induction of UDP-GT activity in NPE and PE cells by PB. Key: (●) and (▲) basal activities of NPE and PE cells respectively; (○) and (△) induced levels of NPE and PE cells respectively; (⊙) and (⊛) inhibition of induced activity of NPE cells by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively; and (◐) and (◭) inhibition of induced activity of PE cells by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively. Bars indicate standard deviations (N = 4).

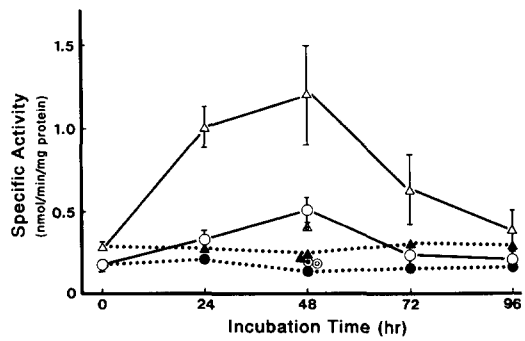

Fig. 6. Induction of UDP-GT activity in NPE and PE cells by MC. Key: (●) and (▲) basal activities of NPE and PE cells respectively; (○) and (△) induced levels of NPE and PE cells respectively; (⊙) and (◐) inhibition of induced activity of NPE cells by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively; and (△) and (◭) inhibition of induced activity of PE cells by 3.5 μM cyclohexamide and 40 nM actinomycin D respectively. Bars indicate standard deviations (N = 4).

cells was 3.8-fold over the basal activity. The induced activity of PE cells was 2 times higher than that of NPE cells. Induction of UDP-GT activity by PB in PE and NPE cells was suppressed almost completely by 3.5 μM cyclohexamide and 40 nM actinomycin D. Figure 6 shows the time course of induction by MC of UDP-GT activity in PE and NPE cells. The results were similar to those of PB treatment; induction of UDP-GT was more pronounced in PE cells than in NPE cells. The induced level of transferase in 48 hr of incubation was 2-fold higher in PE cells than in NPE cells. The enzyme induction was inhibited by 3.5 μM cyclohexamide and 40 nM actinomycin D.

DISCUSSION

Burke *et al.* [6] have described that selective O-dealkylation of the alkyl derivatives of phenoxyazone, ER and PR, is catalyzed by specific cytochrome P450 isozymes. Namely, ER *O*-dealkylase is associated specifically with the MC-inducible isozyme (cytochrome $P450_{MC}$) and PR *O*-dealkylase is linked specifically to the PB-inducible isozyme (cytochrome $P450_{PB}$). Therefore, induction of PR *O*-dealkylase by PB and induction of ER *O*-dealkylase by MC in primary cultures of NPE cells indicate that specific cytochrome P450 isozymes were induced by these compounds in NPE cells. This was supported by the results that anti-cytochrome $P450_{PB}$ antibodies inhibited the PR *O*-dealkylase activity, but not the ER *O*-dealkylase activity, of PB-treated NPE cells and that anti-cytochrome $P450_{MC}$ antibodies suppressed the ER *O*-dealkylase activity, but not the PR *O*-dealkylase activity, of MC-treated NPE cells. Although the presence of cytochrome P450 isozymes in ciliary epithelial cells was not directly demonstrated in this work, induction of cytochrome P450 in the ciliary epithelium of PB-treated rabbit has been shown by immunofluorescence techniques [11]. It awaits further studies to characterize cytochrome P450 isozymes and determine their cellular concentrations in inducer-treated primary cultures of porcine ciliary epithelial cells.

One of the most significant findings in this work is that Phase I enzyme activities, i.e. ER and PR *O*-dealkylase activities, are primarily associated with, and induced by, inducers in NPE cells and not PE cells, and that Phase II enzyme activity, i.e. UDP-GT activity, is distributed and induced by PB and MC in both NPE and PE cells. In other words, Phase I and Phase II enzymes are heterogeneously distributed in ciliary processes. A heterogeneous distribution of drug-metabolizing enzymes in hepatocytes isolated from different regions was reported [12]. The basal and induced levels of 7-ethoxycoumarin *O*-deethylase activity were higher in hepatocytes from the perivenous region than in those cells from the periportal region, while UDP-GT activity did not show regional differences. The heterogeneous distribution of drug-metabolizing capacities was considered to account for the regional differences in the susceptibility of liver cells to toxic chemicals.

What is the significance of the heterogeneous distribution of drug-metabolizing enzyme activities in the ciliary epithelium? A major function of the ciliary epithelium is the formation and secretion of aqueous humor. According to the hypothesis proposed by Cole [13], NPE cells absorb sodium ions from the stroma and pump them out into the intercellular space by active transport (i.e. Na^+, K^+-ATPase). Regional differences in the distribution of Na^+, K^+-ATPase in rabbit ciliary epithelium are known [14]; the NPE cells stain for the enzyme but the PE cells do not. Hyperosmolarity develops in the intercellular space, thereby facilitating the osmotic flow of water from the stroma. Since the tight junctions between NPE cells prevent passage of large molecules, the aqueous humor thus produced is essentially free of proteins. Drugs and environmental chemicals are usually hydrophobic and are able to pass through cell membranes. Hence, the tight junctions cannot prevent their entry into NPE cells and into the intercellular space between

NPE cells. Since drugs and other xenobiotic chemicals may have detrimental effects on the lens and cornea, it is important to minimize the possibility of contaminating the aqueous humor with these chemicals. Polycyclic compounds generally show high affinity for melanin granules of the eye [15]. It is therefore postulated that the melanin granules of PE cells play an important role as the primary defense mechanism and detoxify blood plasma prior to its passage to NPE cells for being processed as the aqueous humor. Phenolic substances may be detoxified by Phase II enzymes such as UDP-GT in PE cells. If the concentrations of drugs and chemicals in blood plasma are so high as to exceed the adsorption capacity of melanin, however, contamination of the aqueous humor by these chemical compounds becomes a serious problem. It is proposed that the major role of the drug-metabolizing enzymes in NPE cells would be to serve as the secondary defense mechanism and remove the xenobiotics by metabolic detoxification.

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