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Effects of polychlorinated biphenyls on cholesterol and ascorbic acid metabolism in primary cultured rat hepatocytes

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We previously reported that dietary polychlorinated biphenyls (PCBs*) produced hypercholesterolemia, and increased urinary and tissue levels of ascorbic acid accompanied the induction of drug metabolizing enzymes in rats [1]. A diet of PCBs or other xenobiotics served to stimulate incorporation of ^3H_2O into liver cholesterol together with an increase in the activity of liver HMG-CoA reductase (EC 1.1.1.34), suggesting that cholesterol synthesis was encouraged [2, 3]. Horio and Yoshida [4] reported that UDP-glucuronyl transferase (EC 2.4.1.17) activity was increased by PCBs or other xenobiotic feeding accompanied by a marked increase in urinary and liver ascorbic acid levels in rats. The precise mechanisms by which PCBs induced these changes related to cholesterol and ascorbic acid metabolism is not yet well understood.

Recently, primary cultured hepatocytes have been widely used in metabolic studies [5, 6]. One of the advantages of this model is to allow the study of metabolic expression of liver cells in a culture medium of defined composition, independently of hormonal or nutritional variations. To our knowledge, no one has reported the effects of PCBs on cholesterol and ascorbic acid metabolism in primary cultured hepatocytes. Thus, the present study was designed to investigate the influence of PCBs on cholesterol and ascorbic acid metabolism by the rat liver parenchymal cells in primary culture.

Materials and Methods

Parenchymal hepatocytes were isolated from adult male Wistar rats, weighing about 200 g, by perfusion of the liver *in situ* with collagenase, essentially as described by Seglen [7]. The viability of cells after isolation as measured by the ability of the cells to exclude trypan blue was usually greater than 90%. The culture medium was Williams medium E supplemented with 10% fetal bovine serum, 10^{-9} M insulin, 10^{-8} M dexamethasone, penicillin 100 units/mL, streptomycin 100 μg/mL and fungizone 0.25 μg/mL. Inocula of 1×10^5 cells/0.2 mL/cm^2 were seeded into collagen-coated petri dishes (Corning, 10-mm plate), and cultured as monolayers in a humidified incubator at 37° under 5% CO_2 in air. The medium was changed after the first 4 hr, and then 6 μM PCB (Aroclor 1248, Mitsubishi Monsanto, Tokyo) was added to the medium after 24 hr in culture, and cells were harvested with a rubber policeman at 48 hr. The composition of Aroclor 1248 [8] is 2% dichlorobiphenyl, 18% trichlorobiphenyl, 40% tetrachlorobiphenyl, 36% pentachlorobiphenyl and 4% hexachlorobiphenyl. The dosage (6 μM) of PCBs was based on previous pharmacological studies [9, 10] and preliminary experiments. PCBs were first dissolved in small amounts of dimethylsulfoxide, and this solution was then added to the growth medium as described previously [10]. Cell number was analysed by the method of Nakamura *et al.* [11]. Cells were incubated in Hanks solution containing 5 mM NH_4Cl and the amount of urea formed was measured by the method of Geyer and Dabich [12]. A sample of 5×10^6 cells was homogenized in 0.4 mL of 5 mM phosphate buffer (pH 7.8) containing 5 mM 2-mercaptoethanol and centrifuged, and the supernatant was used for the assay of tyrosine transaminase (EC 2.6.1.5) activity as described previously [13]. Protein was determined by the method of Lowry *et al.* [14]. Cellular ascorbic acid was measured by 2,4-dinitrophenylhydrazine method [15]. Cellular cholesterol content was measured by an enzymatic colorimetric method (Monotest Cholesterol, CHOD-PAP-method, Boehringer Mannheim GmbH, Mannheim, F.R.G.). HMG-CoA reductase activity was measured according to the method of Ide *et al.* [16]. Cell monolayers were washed twice with cold Dulbecco's phosphate-buffered saline before they were gently scraped from the dishes and centrifuged at 1000 *g* collected in homogenizing tubes in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.25 M sucrose, 0.075 M nicotinamide, 2.5 mM EDTA and 1 mM dithiothreitol. Cells were homogenized with Polytron. Cell homogenates were centrifuged at 10,000 *g* for 20 min. The supernatants were then recentrifuged at 105,000 *g* for 60 min. Microsomal pellets were used for the assay of HMG-CoA reductase activity. Aryl hydrocarbon hydroxylase (EC 1.14.14.2) activity was measured according to the method of Murakami *et al.* [9] and Nebert and Gelboin [17]. Cell monolayers were washed twice with cold 0.05 M Tris–HCl (pH 7.4) containing 0.15 M NaCl before they were gently scraped from the dishes and centrifuged at 1000 *g* collected in homogenizing tubes in 0.05 M Tris–HCl (pH 7.4) containing 0.25 M sucrose. Cells were homogenized with a Polytron. Microsomal pellets were used for the assay of aryl hydrocarbon hydroxylase activity. UDP-glucuronyl transferase activity was analysed according to Henderson [18]. The cell pellets were collected in cold Dulbecco's phosphate-buffered saline and homogenization was carried out in 0.25 M sucrose/0.05 M Tris–HCl (pH 7.5) with a Polytron. The methods for the preparation of microsomal pellets used were the same as those for aryl hydrocarbon hydroxylase. Microsomal pellets were used for the assay of UDP-glucuronyl transferase activity. For the incorporation of [1−^{14}C]acetate into hepatocyte cholesterol, freshly isolated hepatocytes were cultured for 24 hr under standard conditions, and then PCB (6 μM) was added to the medium. After 24 hr incubation, the cells were incubated with 6 mL

* Abbreviations: PCB, polychlorinated biphenyls; HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme A.

of culture medium containing [1-^{14}C]acetate (3 μCi/6 mL) for 3 hr. The cells were washed twice with cold Dulbecco's phosphate-buffered saline and harvested, and then the radioactivity in digitonin-precipitable sterols was analysed by the method as described previously [3].

Data were analysed by Student's *t*-test [19].

Results and Discussion

Urea formation, the cell number, cellular protein contents (data not shown in Table 1) and tyrosine transaminase activity were not changed by PCB addition in cultured rat hepatocytes. The present results indicate that these liver functions are near to control levels despite the addition of PCBs. Cellular ascorbic acid level and UDP-glucuronyl transferase activity were increased significantly by the addition of 6 μM PCB. Horio and Yoshida [4] previously observed a marked increase in UDP-glucuronyl transferase activity induced by PCB *in vivo* and indicated the causal interrelationships between the induction of this enzyme and liver ascorbic acid level. We also speculated that the increase in liver level of ascorbic acid *in vivo* was caused by a stimulation of ascorbic acid biosynthesis in the liver [4]. The present results suggest that these metabolic changes related to ascorbic acid induced by dietary PCB *in vivo* were also observed in primary cultured rat hepatocytes. Our preliminary observations indicate that the ascorbic acid level in culture medium is also increased by PCB, accompanying an increase in cellular level of ascorbic acid. Hence, these findings indicate a possibility that the ascorbic acid accumulation in cultured hepatocytes caused by PCB is mainly attributable to the stimulation of cellular ascorbic acid synthesis mediated by the increment of UDP-glucuronyl transferase activity in the D-glucuronic acid pathway (ascorbic acid-synthesizing pathway).

PCBs caused an increase in aryl hydrocarbon hydroxylase activity in rat hepatocytes as observed *in vivo*. The present results reveal that aryl hydrocarbon hydroxylase activity is increased by PCBs, accompanied by an increase in the hepatocyte ascorbic acid level. We have already suggested that the simultaneous effect of xenobiotics such as PCB on drug-metabolizing enzymes and liver ascorbic acid [1]. Similar observations were obtained in the present studies.

Our previous reports [2, 3] have indicated that the incorporation of $^{3}H_2O$ into hepatic cholesterol and hepatic HMG-CoA reductase activity were significantly higher in rats fed PCBs- or other xenobiotic-containing diets.

The incorporation of [^{14}C]acetate into hepatocyte cholesterol was significantly increased by the addition of 6 μM PCB in the cultured system, but the increase is very small. In cultured cells, HMG-CoA reductase activity was not changed at 24 hr PCB-induction. The lack of response of HMG-CoA reductase observed in the hepatocytes may be due to the fact that exogenous cholesterol added in the foetal calf serum component of the culture medium is inhibiting this enzyme activity. But, Edwards [20] has already suggested that the serum elicited an increase in the activity of HMG-CoA reductase and the rate of sterol synthesis. Thus, it is unclear whether or not exogenous cholesterol added in the foetal calf serum component of the culture medium is inhibiting the HMG-CoA reductase activity. Our preliminary observations suggest that there are no significant changes in cholesterol contents of culture medium and the incorporation of [^{14}C]acetate into cholesterol secreted into the culture medium with or without PCBs. Moreover, the cellular cholesterol level was also unchanged by PCB addition. But, *in vivo*, PCBs caused an increase in liver cholesterol content as shown previously [1]. Why there are differential effects of PCBs on cholesterol metabolism between *in vivo* and *in vitro* remains unclear at present. Thus, to reproduce the alterations induced by PCBs on cholesterol metabolism as observed *in vivo*, it may be necessary to consider unknown factors in primary cultured systems. Our results show that primary cultured hepatocytes mimic partially the intact liver with regard to some responses to PCBs. Our previous [3, 4] and present results may suggest that the metabolic changes in relation to ascorbic acid *in vivo* induced by dietary PCBs were primarily related to the direct effects of PCBs *per se* on liver parenchymal cells rather than the changes in hormonal status induced by PCBs. Studies are now in progress to obtain further information on the mechanisms of PCB-induced changes in cholesterol and ascorbic acid metabolism.

In summary, urea formation, cell number, cellular protein content and tyrosine transaminase activity were not changed by PCB addition in cultured rat hepatocytes. These

Table 1. Effects of PCBs on urea formation, cellular ascorbic acid and cholesterol contents, tyrosine transaminase, aryl hydrocarbon hydroxylase, HMG-CoA reductase and UDP-glucuronyl transferase activities and the incorporation of [1-^{14}C]acetate into hepatocyte cholesterol in primary cultured rat hepatocytes

	Control	PCB(6 μM)
Urea formation (nmol/mg cellular protein/hr)	484.5 ± 8.6	469.8 ± 8.6
Cellular ascorbic acid (ng/mg cellular protein)	223.0 ± 3.0	608.5 ± 6.5‡
Cellular cholesterol (μg/mg cellular protein)	37.7 ± 1.5	35.5 ± 0.8
Tyrosine transaminase (nmol/min/mg protein)	3.70 ± 0.01	3.42 ± 0.04
Aryl hydrocarbon hydroxylase (pmol/min/mg protein)	35.9 ± 5.8	150.4 ± 1.8‡
HMG-CoA reductase (pmol/min/mg protein)	1.22 ± 0.15	1.20 ± 0.27
UDP-glucuronyltransferase (nmol/min/mg protein)	4.90 ± 0.62	6.65 ± 0.14*
[^{14}C]acetate incorporation into hepatocyte cholesterol (dpm/mg cellular protein/hr)	5544 ± 101	5932 ± 72†

Values are means ± SEM of four separate experiments.

Statistical significance compared with control group (* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$).

results indicate that these liver functions are similar to control levels despite the addition of PCBs. Our results show that primary cultured hepatocytes mimic partially the intact liver in relation to some responses to PCBs. In isolated hepatocytes as well as *in vivo*, PCBs increased in cellular ascorbic acid level, aryl hydrocarbon hydroxylase and UDP-glucuronyl transferase activities. Thus, the present results may suggest that the metabolic changes *in vivo* concerning ascorbic acid induced by dietary PCBs were primarily related to the direct effects of PCBs *per se* on liver parenchymal cells.

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Immunoreactivity of endogenous digitalis-like factors

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Much evidence supports the view that the natural ligands of the digitalis receptor of the Na^+, K^+-ATPase may exist in the mammalian body. Such endogenous digitalis-like factors (EDLFs) may be physiological regulators of the enzyme and may play important roles in the regulation of sodium excretion and the pathophysiology of hypertension [1, 2]. Many investigators have chosen to use radioimmunoassay for digoxin to determine EDLFs on the assumption that an endogenous compound binding to a specific receptor may also bind to an antibody raised against the exogenous ligand [3]. However, this approach has many problems and has been a source of much controversy [4].

We have emphasized the digitalis-like biological activities to search for the EDLFs and were able to isolate two distinct EDLFs from human urine based on an inhibitory effect on [^{3}H]ouabain binding to intact human erythrocytes