A COMPARISON OF THE CELLULAR UPTAKE OF POLYCHLORINATED BIPHENYLS IN DIVERSE CULTURED ADIPOCYTE MODELS

Bourez S¹, Van Den Daelen C.¹, Louis C¹, Le Lay S³, Larondelle Y¹, Remacle C¹, Thomé JP², Schneider YJ¹, Dugail I³, Debier C¹

¹ Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud, B-1348 Louvain-la-Neuve, Belgium ; ² Laboratoire d'Ecologie animale et d'Ecotoxicologie. Université le Liège. Allée du 6 Août, 15, B-4000 Liège, Belgium ; ³ Centre de Recherche des Cordeliers, INSERM, U872, Paris, France

Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental pollutants that tend to accumulate in lipid-rich tissues of the organisms they contaminate, due to their highly lipophilic properties. The adipose tissue therefore constitutes one of the most significant intern reservoirs of such organic pollutants¹. Recent epidemiological studies suggested a role for these chemicals in the fundamental mechanisms controlling the regulation of the energetic balance². PCBs could indeed potentially be involved in the obesity epidemic. Moreover, it has been shown that during periods of body weight loss, PCB concentration increases in the adipose tissue and in plasma due to lipid mobilization (reviewed in 2). As both circulating and tissue concentrations are then higher, potentially exposing the individual to the various known adverse health effects of these pollutants^{3,4}. However, few data are available about the mechanisms involved in the storage and release of lipophilic pollutants such as PCBs in/from the adipose tissue. In addition, there is a lack of simple in vitro test models of adipocytes, available for the characterization of accumulation kinetics, storage and release of PCBs and related molecules as well as the screening of potential toxicological effects of such pollutants in this endocrine tissue. In the present study, we evaluated the accumulation rate of a cocktail of 3 PCB congeners, 2.4,4'-trichlorobiphenyl (PCB-28), 2,3',4,4',5-pentachlorobiphenyl (PCB-118) and 2,2',4,4',5,5'-hexachlorobiphenvl (PCB-153) added in the medium of three classically used in vitro models of adipocytes: mouse embryonic fibroblasts (MEFs) differentiated into adipocytes, primary cultures of rat adipocytes and the 3T3-L1 preadipocyte cell line.

Materials and methods

Cell culture.

- 3T3-L1 cells (kind gift of Dr J. Pairault, Paris, France) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) until 2 days post-confluence (day 0). Adipocyte differentiation was induced by adding isobutylmethylxanthine (IBMX, 250 μ M), dexamethasone (Dex, 1,25 μ M), insulin (250 nM), penicillin (100U/ml) and streptomycin (0.1 mg/ml) for 2 days. The cells were then cultured for 2 more days in the medium just described but without IBMX and Dex and with lower insulin concentration (100 nM). For the rest of the differentiation process, insulin was also suppressed from the medium which was then replaced every 48h until 10-12 days post-confluence for experiments.

- MEFs were prepared as described in (5). Adipocyte differentiation was induced during 2 days as described for 3T3-L1 cells except for IBMX (500 μ M) and insulin (870 nM) initial concentrations. For the rest of the differentiation process, IBMX and Dex were removed and rosiglitazone (0,5 μ M) was added to the differentiation inducer cocktail every 48h during 6-7 days.

- Preadipocytes were isolated from perigonadal adipose tissue as described in (5). The preadipocyte cells were cultured at 37°C under a 10% CO₂ atmosphere for 24h in DMEM containing 4.5 g/l glucose, glutaMAX, and pyruvate supplemented with 10% (v:v) fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μ g/ml), amphotericin B (250 ng/ml), gentamycin (500 ng/ml) and nystatin (40 U/ml). Then, the medium was changed to a differentiation medium containing DMEM with the same components (without gentamycin and nystatin), and 10 μ M of ciglitizone, 8.5 μ M of insulin and 10 nM of dexamethasone were added. The

differentiation medium was changed every 48 h and preadipocytes were cultured for 13 days after inoculation for experiments.

- Once the differentiation was achieved, the cells from the 3 adipocyte models were incubated during 4 hours at $37^{\circ}C - 10\% CO_2$ with a cocktail of 3 PCB congeners, 2,4,4'-trichlorobiphenyl (PCB-28); 2,3',4,4',5-pentachlorobiphenyl (PCB-118) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153). Longer incubations were tested for the 3T3-L1 adipocytes (up to 8 hours) and for the primary cultured cells (up to 48h) as we obtained more plates per isolation as compared to MEFs. PCBs final concentration (50 ng/ml for each PCB congener) was applied as an ethanolic solution in the medium. Control cells received the ethanol vehicle alone that did not represent more than 0,5% vol.

Biochemical and chemical analyses.

PCBs were analyzed by gas chromatography using a Thermo Quest Trace 2000 gas chromatograph equipped with a 63 Ni ECD detector. Details of the procedure are described in (6). Total cellular glycerolipids were measured, after the chemical saponification of the cell lysates in a 0.1M KOH-MeOH solution for 1h at 70 °C, via the quantification of released glycerol using a commercially available kit (Free glycerol FS, DiaSys). Phospholipids (PL) were quantified by gas chromatography and enabled to calculate the amounts of triglycerides (TG) present in the cells. Protein contents were determined using the Bicinchoninic Acid (BCA) protein assay kit (Sigma-Aldrich). Cytotoxicity of the PCBs was assessed by measuring the activity of lactate dehydrogenase (LDH) released into the extracellular medium of treated cells, using the Cytotoxicity Detection Kit (Roche diagnostics, Mannheim, DE) according to the manufacturer's instructions, using 1% (v/v) Triton X-100 as full toxicity control. Cell morphology was also daily observed by phase contrast microscopy.

Results and discussion

Control of PCBs recovery in our experiments

PCBs being highly lipophilic molecules, it was important to first verify that they did not significantly adsorb to the plastic materials used in our assays. We therefore summed up the amounts of PCBs quantified in the media and cells for each time point of the incubation. Total quantities of PCBs fairly corresponded to the initially added amount (within error bars), showing that there was no significant loss of PCBs by adsorption on culture materials during the incubation period. The presence of 10% foetal bovine serum (FBS) in our culture media which is rich in bovine serum albumin (BSA) and lipoproteins, is most probably the reason for the sequestration of PCBs in the medium rather than on the plastic materials. Indeed, the data obtained with non FBS-containing media revealed important adsorptions of PCBs to plastics (results not shown). These recoveries were tested in all our experiments and showed the same results.

Accumulation pattern of PCBs-28, 118 and 153 in 3 adipocyte models

After 4 hours of incubation, the percentage of accumulation of PCB-28 from a same initial amount added in the medium sharply differed among the adipocyte models, from 23% in MEFs, to 64% in primary cultures and up to 82% in 3T3-L1 adipocytes (Figure 1A, 1B, 1C). Similar observations were made with PCBs-118 and -153 for which accumulation rates were respectively 13 and 13 % in MEFs, 43 and 28 % in primary cultures and 82 and 76 % in 3T3-L1 adipocytes (Fig. 1D-I). Interestingly, the quantification of the cellular levels of triacyglycerols (TG) also showed an important difference between the cell models, ranging from $4 \pm 1 \mu g/well$ in MEFs, to $10 \pm 2 \mu g/well$ in primary cultures and up to $24 \pm 2 \mu g/well$ in 3T3-L1 adipocytes. The amounts of accumulated PCB-28, -118 and -153 were highly correlated to the cellular levels of TG in each adipocyte model (Table 1). The total amounts of PCBs (expressed per unit of TG) accumulated in each model was $3 \pm 1 ng/\mu g$, $5 \pm 2 ng/\mu g$ and $2 \pm 1 ng/\mu g$ for MEFs, primary cultures and 3T3-L1 adipocytes respectively. These results show that, when expressed per unit of cell TG, total amounts of PCBs accumulated after 4 hours of incubation are in the same range of values in all tested adipocyte models.



Figure 1- Accumulation rate of PCB-28 (A, B, C), PCB-118 (D, E, F) and PCB-153 (G, H, I) added during 4 hours as a cocktail (final concentration in the medium of 50 ng/ml each) on MEFs differentiated into adipocytes (A, D, G), primary cultures of differentiated rat adipocytes (B, E, H) and differentiated 3T3-L1 cells (C, F, I).

1 able 1	rearson contraction coefficients between central inplus and reds-20, 110 and 195.			
		PCB-28	PCB-118	PCB-153
	r	0.960	0.983	0.937
	P-value	< 0.01	< 0.01	< 0.01

Table 1 – Pearson correlation coefficients between cellular lipids and PCBs-28, 118 and 153.

Stabilization of the accumulation is not the result of a restriction of PCBs in the medium

As clearly shown in the MEFs model, PCB-28 accumulation in the cells reached a plateau after 4 hours of incubation. The same results were obtained in the primary cultures where accumulation rates of PCB-28 did not significantly increase anymore between 4 and 48h of incubation with the pollutants ($64 \pm 3\%$ and $70 \pm 1\%$ respectively). This phenomenon was however not the result of a PCB restriction in the media of the cells as 80% and 36% of PCB-28 were still present in the media of MEFs and primary cultures. Moreover, in each of the tested models, PCB-28 entered the cells more rapidly than the two other congeners. Indeed, after only 1h of incubation, 20 \pm 5 % of PCB-28 had accumulated in the cells against only 6 ± 2 % for PCB-118 and $5 \pm 2\%$ for PCB-153. In primary cultures, accumulation rates after the first hour were in the same order, namely $32 \pm 4\%$ for PCB-28, $13\pm$ 3% for PCB-118 and $7 \pm 2\%$ for PCB-153. In the 3T3-L1 adipocytes, that contained higher amounts of TG, we

observed accumulation rates reaching $49 \pm 5\%$ for PCB-28, $32 \pm 3\%$ for PCB-118 and $28 \pm 1\%$ for PCB-153 after only 1 hour of incubation with the pollutants. In all tested models, the entrance of PCBs in adipocytes during the first hours of incubation always respected the following order: PCB-28 > PCB-118 > PCB-153. As a consequence, it seems that the number and probably the position of the chlorine substituents on the biphenyl backbone of the PCBs structure determined their dynamics of accumulation in adipocytes.

Adipocytes concentrate PCBs present in the surrounding medium

We calculated the concentration factor of each PCB congener between the cell layer and the initial concentrations added to the medium. The cell layer of one experimental unit (10cm^2) was estimated at 5 µl whereas the corresponding growth medium volume was 2 ml. After 4 hours of incubation, adipocytes concentrated PCB-28 by about 95 times in the MEF model, 260 times in the primary cultures and up to 340 times in the 3T3-L1 condition. In both MEFs and primary cultures where a plateau was reached after 4 hours of incubation, the concentration factors of PCB-28 between the cells and the surrounding medium were higher than for the two other congeners (53x and 52x for PCBs 118 and 153 in MEFs and 177x and 114x for PCBs-118 and -153 in primary cultures). In 3T3-L1 adipocytes where no plateau was observed after 4 hours of incubation, all concentration factors were fairly similar for all congeners (341x, 337x and 313x for PCB-28, -118 and -153 respectively).

Taken together, these results show that adipocytes are capable of concentrating massive intracellular amounts of lipophilic pollutants such as PCBs. Those in vitro culture models are useful tools to study the uptake potential of fat cells for lipophilic compounds and to evaluate both qualitative and quantitative aspects of their kinetics and toxicity in the adipose tissue.

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