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Differential Transfer of Organic Micropollutants through Intestinal Barrier Using Caco-2 Cell Line

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Food seems to be one of the main ways of animal and human contamination with polycyclic aromatic hydrocarbons (PAHs) and dioxins. In vivo studies showed a blood absorption of these xenobiotics after their ingestion. Our work aimed at studying the in vitro transfer of PAHs and dioxins through intestinal barrier. Caco-2 cells were cultivated on permeable filters to measure transepithelial permeability of ^{14}C labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, benzo[*a*]pyrene, pyrene, and phenanthrene, which differed in their physicochemical properties. The results showed that the molecules were able to cross intestinal cell layers. All the molecules were detected associated with cells, even if the dioxin was the less uptaken compound. Phenanthrene appeared in basal media faster, and its level after a 6-h exposure was respectively 1.1, 2, and 7 times higher than pyrene, benzo[*a*]pyrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin levels. These findings suggest that intestinal epithelium plays a key role in selective permeability and then in bioavailibility of micropollutants.

KEYWORDS: Caco-2 cells; PAHs; dioxin; intestinal epithelium; transfer

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) and dioxins are ubiquitous organic molecules released in appreciable amounts in the environment (*1*−*4*). Their hydrophobic character (*n*-octanol/water partition coefficients, log $K_{ow} > 4$) and their high resistance to biodegradation and elimination give them a tendency to accumulate in food chains, resulting in high and toxic levels in organisms of higher tropic levels and humans (*2*, *4*−*8*).

Diet is one of the main sources of human and animal background exposure to these lipophilic organic pollutants (*9*). Gastrointestinal absorption of these compounds plays a key role in determining how the dietary exposure effectively translates into tissue.

Little is known about factors governing intestinal absorption of these molecules in humans or animals. Studies in vivo with fish, rats, or pigs proved that PAHs are differently absorbed by the gastrointestinal tract probably according to their physicochemical properties (*10*−*14*).

In recent studies, cultures of Caco-2 cell monolayers, isolated from a human colon carcinoma, have been used as a model system to study the intestinal uptake and transport processes of hydrophobic xenobiotics such as polychlorinated biphenyls (*15*, *16*). Differentiated post-confluent Caco-2 cells exhibit well-developed microvilli, and grown on permeable supports, they form tight monolayers with a polarized distribution of brush border enzymes (*17*).

Table 1. Physicochemical Properties of the Studied Organic Micropollutants

compd	fused benzene ring no.	lipophilicity log K_{ow}	water solubility (mg/L)	mol wt (Da)
TCDD	2	6.8	1.93×10^{-5}	321.98
BaP	5	6.31	3.80×10^{-3}	252.00
Pyr	4	4.88	1.30×10^{-1}	202.3
Phe	3	4.5	1.21	178.2

The study of Artursson and Karlson (*18*) confirmed the capability of these cells in trans-cellular transport of hydrophobic drugs such as felodipine and testosterone (log K_{ow} of 3.48 and 3.31, respectively). Wils et al. (*19*) showed that highly lipophilic molecules are more slowly transported across epithelia. Apart from physicochemical properties, this phenomenon seems related to the physiology of epithelial cells (apical efflux system and metabolism). It suggests that the intestinal epithelium is the most important barrier to the absorption of lipophilic molecules.

In the present study, we used the Caco-2 cell line to study the uptake and transport of ^{14}C-labeled organic micropollutants: one dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)) and three PAHs, chosen with increasing number of cycles and decreasing water solubility (benzo[*a*]pyrene (BaP), pyrene (Pyr), and phenanthrene (Phe)) (**Table 1**).

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MATERIALS AND METHODS

Cell Culture. Basal defined medium (BDM) was composed of Iscove's modified Dulbecco's medium, F 12 nutrient HAM, NCTC-135 medium (5/5/1) purchased by Invitrogen (Cergy Pontoise, France), glutamine (6 mM), sodium bicarbonate (4 g/L), and ethanolamine (50 mM). These medium components, antibiotics, hydrocortisone, epidermal growth factor (EGF), triiodothyrosine, insulin, linoleic acid albumin, dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS) came from Sigma Aldrich (Saint Quentin Fallavier, France). Fetal calf serum (FCS) was from Biowest (Nuaille, France). Collagene solution was from Cellon (Luxemburg), trypsine (2.5 g/L)−EDTA (1.1 g/L) solution was from Biowhittaker (Emerainville, France), and nonessential amino acids were from Invitrogen (Cergy Pontoise, France). Cell culture inserts were supplied by Merck Eurolab (Fontenay sous Bois, France). Caco-2 cells were obtained from Cellular Biochemistry Laboratory (Louvain-la-Neuve University, Belgium). Cells from passage 210−225 were used in the experiments.

The cell line was maintained in BDM containing 1% nonessential amino acids, insulin (1 μg/mL), penicillin (100 U/mL medium), streptomycin (100 μg/mL), gentamycin (100 μg/mL), hydrocortisone (0.1 μM), EGF (1 ng/mL), triiodothyrosine (2 nM), insulin (1 μg/mL), and linoleic acid albumin (10 μg/mL) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Cells were routinely frozen in culture medium containing 20% FCS and 10% DMSO. Cells were seeded on filter inserts (4.2 cm^2, 1 μm pore size) at a cell density of about 160 000 cells/cm^2 and grown for 17 days. Medium was changed every 2 days.

Before transport studies assessment, integrity of cell monolayers was first assessed by measuring the transepithelial electric resistance with a Mitchell-ERS Epithelia voltohmmeter (Millipore Co., Bedford, MA). Monolayers of cells showed a mean transepithelial resistance of 692 (sd = 21, $n = 48$) $\Omega \cdot cm^2$. These resistances verified epithelia integrity (*20*, *21*).

Preparation of PAHs and TCDD-Containing Media. The four compounds were chosen according to their different physicochemical properties (**Table 1**). U-^{14}C-TCDD (45.4 mCi/mmol) was purchased from Isobio (Belgium), 7,10-^{14}C-BaP (50 mCi/mmol) was from Amersham (Buckinghamshire, England), 4,5,9,10-^{14}C-Pyr (58. 7 mCi/mmol) was from Sigma Aldrich (Saint Quentin Fallavier, France), and 9-^{14}C-Phe (55 mCi/mmol) was from Moravek Biochemicals (Brea, USA).

Every contaminated culture medium contained 3.4×10^{-2} μCi/mL. The compounds were dissolved in DMSO and methanol (Sigma Aldrich, Saint Quentin, France). Methanol and DMSO final concentrations in contaminated and control culture media were respectively 5×10^{-4} and 0.1%, as Carriere et al. (*22*) ensured that this last concentration had no effect on any parameter of such study (cell viability, epithelium integrity).

Incubation of Cells with PAHs and Transfer Analysis. All experiments were conducted at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. After 17 days of culture, the apical side received contaminated or control medium. Exposures were continued for 15, 90, 180, and 360 min. Four repetitions were achieved for each point. At each time and for each molecule, 500 μL of basal medium of four culture wells were sampled in triplicate, added with 10 mL of Ultimagold (Packard, Rungis, France) scintillation liquid and counted for 10 min using a Tricarb 460 CD liquid scintillation counter (Packard, Rungis, France). Cells were rinsed with water, scrolled, and collected in a solution of soluene (Packard, Rungis, France) and water (8/2). The mixture was kept at 50 °C for 2 h and was eventually counted in liquid scintillation with Hionic Fluor (Packard, Rungis, France). Results were expressed in percentages of the radioactivity dose brought in apical medium.

Statistical Analysis. Percentages of radioactivity counted in basal medium and in cells were analyzed with the software STATBOX (Gremmer software) by variance analysis in total randomization. The model included 2 factors: one factor time (4 independent modalities: 15, 90, 180, and 360 min; each time measure was realized with different wells), one factor molecule (4 independent modalities: TCDD, BaP, Pyr, and Phe) and their interaction time × molecule. Four repetitions were realized for each point, which means that 4 times × 4 molecules × 4 repetitions = 64 experimental units.

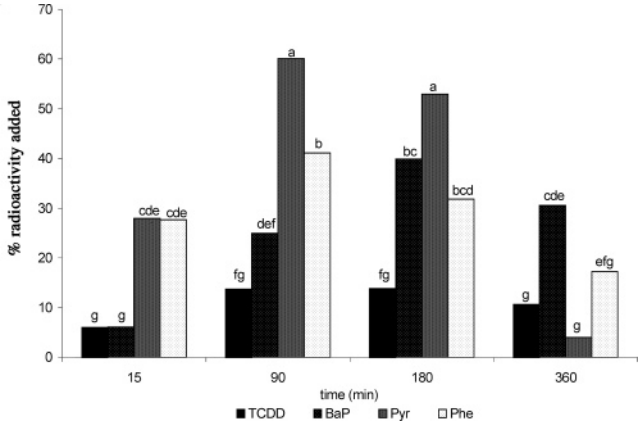

Figure 1. Part of ^{14}C-radioactivity apically added measured bound to cells between 15 and 360 min.

Table 2. Significance of Tested Effects[a]

	effects				
	time	molecule	time × molecule	mean	sme
% radioactivity associated with cells	$P < 0.001$	$P < 0.001$	$P < 0.001$	25.35	6.29

[a] $n = 64$ (time 4, molecule 4, repetition 4).

RESULTS

Controls. *Control Medium.* No radioactivity was detected in filters; apical and basal media of inserts cultivated with control media were added apically. Therefore, radioactivity measured in the tested inserts did come from contaminated media only.

Permeable Membrane. Transport of the micropollutants through permeable membrane without cell monolayer appeared to be fast. The levels of measured basal radioactivity reached about 3−10% as soon as 15 min and remained stable all along the kinetics. The filter was then a barrier for the molecules, but cells remained the principal determinant of their transfer.

Adsorption to Insert Walls. About 1−2% of the total bringing radioactivity was recovered from the apical walls cleaning after 6 h. Wash basal walls provided less than 0.1% of apical original radioactivity. These minor amounts would scarcely influence kinetics.

Radioactivity Associated with Intestinal Cells. This study was carried out to measure intestinal cell-associated radioactivities. It allowed the evaluation of the percentage of each molecule that was adsorbed to cell membranes or absorbed in cell cytoplasm (**Figure 1**).

Cell association profiles appeared different according to the molecule studied. TCDD uptake remained stable ($P < 0.001$) and represented 6.1% of the added TCDD at 15 min and reached a steady-state of approximatively 13% as soon as 90 min (**Table 2**). BaP quantity bound to cells increased linearly from 6.1% at 15 min to reach a peak of 39.9% at 180 min. Then, cell radioactivity decreased but was still of 27.3% at the last point. Pyr and Phe was more rapidly removed from culture medium as 28% of their apically added dose was found associated to cells at 15 min ($P < 0.001$). They kept on increasing till 90 min to reach respectively 60.1 and 41.1% and then decreased linearly until 360 min where their values were 4 and 17.3%.

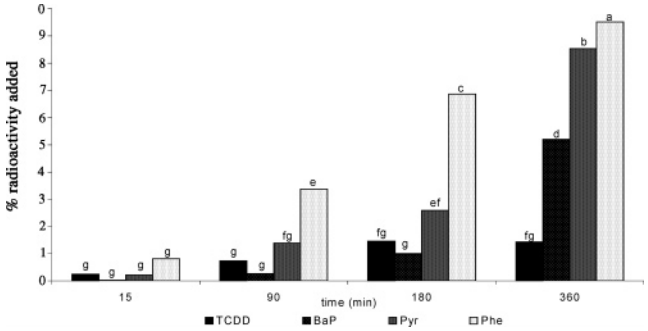

Figure 2. Part of ^{14}C-radioactivity apically brought measured in basal medium between 15 and 360 min.

Table 3. Significance of Tested Effects

	effects				
	time	molecule	time × molecule	mean	sme
% radioactivity detected in basal medium	$P < 0.001$	$P < 0.001$	$P < 0.001$	2.73	0.65

[a] $n = 64$ (time 4, molecule 4, repetition 4).

Adsorption and absorption were the first step needed to cross the barrier and reach the basal side.

Radioactivity Transferring to Basal Compartment. The transepithelial passage of the compounds was then studied on the Caco-2 monolayers. First of all, the studied micropollutants were able to cross the intestinal barrier. Radioactivity was readily observed after a 15-min incubation (**Figure 2**). Regarding the graph, transfer rates were different for the four molecules:

TCDD appeared in very little quantity all along the kinetic. Its basal transfer increased very slowly between 15 and 90 min (0.3 and 0.7%, respectively). Up to 90 min, minor amounts of TCDD were still transported in basal medium, values not exceeding 1.5%. Release of radioactivity originating from BaP in the basal medium seemed initially very low (only 0.0, 0.3, and 1% at 15, 90, and 180 min). However, the detected radioactivity significantly increased after 180 min and reached 5% at 360 min ($P < 0.001$). Pyr showed a similar behavior. Its detected radioactivity remained quite low between 15 and 180 min (0.2, 1.4, and 2.6% at 15, 90, and 180 min), but its level dramatically increased to 8.5% of apical radioactivity which crossed intestinal barrier ($P < 0.001$) (**Table 3**). Phe was significantly more present as soon as 90 min ($P < 0.001$). It was vectorially discharged into basal medium earlier (0.8% at 15 min) and kept on increasing till 360 min where 9.5% of the originated apical dose appeared in the basal side.

DISCUSSION

The present investigation showed a differential absorption and transfer of the four molecules. Pyr and Phe appeared the most and the fastest uptaken compounds. Phe was finally transported into basal side 1.1-, 1.8-, and 6.7-fold more than respectively Pyr, BaP, and TCDD after a 6-h exposure ($P < 0.001$). This differential absorption is similar to a previous in vivo study with pigs where Phe was more detected in plasma than BaP and TCDD after the ingestion of contaminated milk (*23*). TCDD was the less absorbed and excreted molecule.

Comparing to in vivo studies, our results supported the hypothesis that the transfer occurred rapidly. Observations with rats or pigs showed that BaP and Phe appeared in plasma as

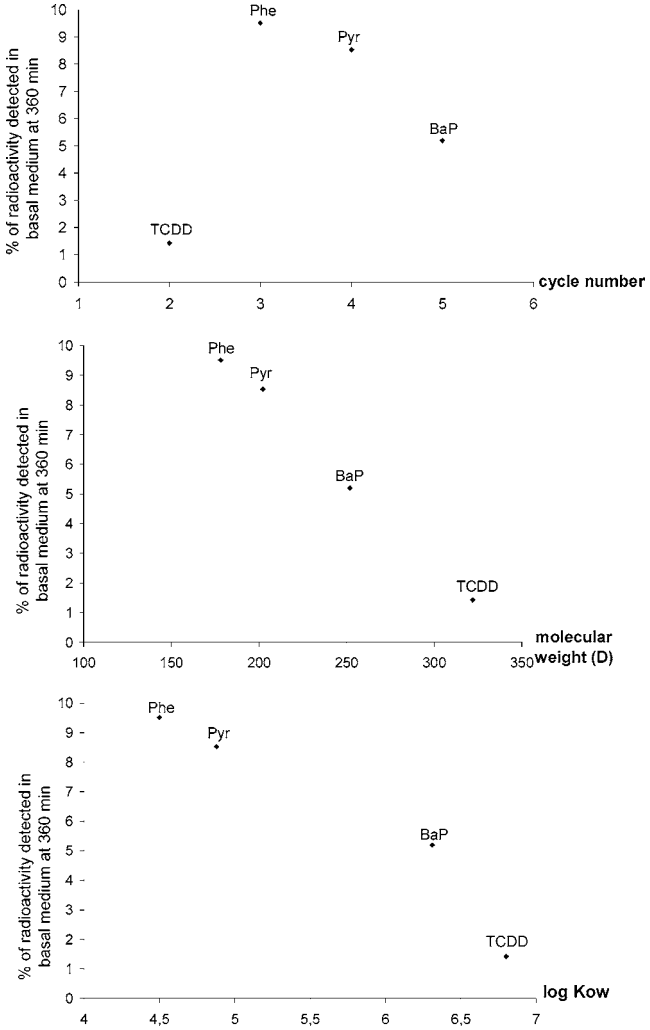

Figure 3. Relation between organic micropollutant part detected in basal medium at 360 min and their physicochemical properties.

soon as 1 h after their oral ingestion (*12*, *14*). BaP intestinal absorption has been explored and estimated at approximatively 20% in an intraduodenal loop and at 30% in the whole gut of rats 18 h after medium removal (*11*, *26*, *27*). Phe absorption has been evaluated at 70% for rats (*11*). Pigs fed with milk contaminated with ^{14}C-Phe, BaP, and TCDD showed an intestinal absorption, determined by the portal blood rate, of 95, 33, and 9%, respectively (*23*).

In vitro proportions between basally transferred Phe, BaP (1.8-fold less detected) and TCDD (6.7-fold less detected) appeared quite similar as in vivo results where Phe was respectively 2.8- and 10-fold more measured in blood than BaP and TCDD (*23*) and proved that the Caco-2 cell line is a good model to study organic xenobiotic transfer.

Micropollutants size might in part play a differential role in the observed transfer. Among PAHs, the quantity of radioactivity measured in basal medium was inversely reliable to their molecular weight and number of cycles (**Figure 3**). But the two- and three-benzene rings of TCDD and Phe suggested that the number of cycles was not a reliable factor to estimate the transfer. Inasmuch their size, it may be argued that lipophilicity and molecular weight could explain the differential intestinal transport too. Phe, the less lipophilic (log $K_{ow} = 4.5$) and the lightest pollutant, had the best transfer; whereas the other compounds saw their passage decreasing in rapidity and quantity as their lipophilicity and molecular weight increased. Study in

situ in rat jejunum involving BaP and naphthalene, which has physicochemical characteristics close to those of Phe, reported similar results (*24*). The mechanism of absorption and transfer of these micropollutants seemed mostly dependent on their physicochemical properties. This is supported by the Vetter et al. (*10*) observations, which showed that intestinal absorption of lipophilic toxicants appeared to be closely related to concomitant dietary fat digestion and absorption. For substances with very low aqueous solubility, the unstirred water layer adjacent to microvillus membrane of the enterocytes created a resistance to absorption, which increases with the molecule lipophilicity (*16*). This resistance is overcome by biliary acids for high lipophilic molecule.

In the present study, BaP and Pyr low transfer at the beginning of the kinetic was not due to a limited absorption because its cell-associated radioactivity was quite effective as soon as 15 min. Their sizes and their lipophilicities could justify their less important transfer but not the delay needed. Previous works supported a PAH metabolization particularly in liver but also in intestinal cells (*10*, *24*). Fish intestinal cells were able to metabolize PAHs thanks to the cytochrome P450 and conjugative enzyme system. It can be envisaged that the two compounds were first segregated and maybe partially metabolized in cells before crossing intestinal epithelium and reached basal side. This is supported by the observation of a consequent level of radioactivity bound to cells between 90 and 180 min, which finally decreased between 180 and 360 min, when the basal transfer increased. Investigations in vivo demonstrated a BaP metabolization in intestinal cells estimating of about 37% in rat jejunum and about 50% after 6 h in killifish gut (*10*, *24*). No such report dealt with pyrene. Van Shooten et al. (*13*) suggested that the intestinal degradation of PAHs and other lipophilic micropollutants, which would aim at detoxifying the toxicants, would turn these molecules into more hydrophilic compounds that better cross the barrier and were mostly eliminated in urine and faeces. Small PAHs with a lower lipophilicity seemed able to cross intestinal barrier rapidly and might not be submitted to a consequent metabolization at this level (*24*).

TCDD reached rapidly a steady-state after 90 min, its level of radioactivity bound to cells and its presence in basal medium remained low and stable. No work dealt with intestinal cells, but it is believed that, even in liver, TCDD was scarcely metabolized (*25*). It might be protected by its steric bulkiness. This confirmed the finding that a part of basal radioactivity originating from BaP, which had a similar lipophilicity than TCDD, would come from its metabolization.

Our experiments showed a differential transfer of the micropollutants in adequation with in vivo observations. The selectivity of intestinal epithelial barrier seems to explain in part the reported differences. Molecule lipophilicity appeared to play a role in their passage quickness and quantity differences too. Many regulatory factors were not present our in vitro model. Biliary acids, present in vivo, have been shown to facilitate the transfer of organic micropollutants by micellar transport (*9*, *13*, *19*). The presence of fatty acids enhanced the absorption of hydrophobic pollutants, such as PAHs or polychlorinated biphenyls too (*8*, *23*).

Further investigations will then focus on transfer to specify its mechanisms (diffusion, transporters, etc.) and the involvement of these different factors. Moreover, the delay, when pollutants are segregated in cells, may be linked to the molecule ability to be metabolized. Our future works will aim at clarifying the possibility of a micropollutant degradation in intestinal cells and its consequences on the transfer too.

ABBREVIATIONS USED

BaP, benzo[*a*]pyrene; BDM, basal defined medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FCS, fetal calf serum; PAHs, polycyclic aromatic hydrocarbons; PBS, phosphate-buffered saline; Phe, phenanthrene; Pyr, pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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