Short Communication: Study of Mammary Epithelial Role in Polycyclic Aromatic Hydrocarbons Transfer to Milk

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ABSTRACT

Food would appear to be one of the main routes for animal and human contamination with polycyclic aromatic hydrocarbons (PAH). Many studies have shown the presence of PAH in milk and dairy products, suggesting that these foods can represent a part of this contamination. Our work aimed at defining, *in vitro*, the mammary barrier role in PAH transfer to milk. MAC T cells were cultivated on permeable filters to measure transepithelial permeability of ¹⁴C labeled benzo[a]pyrene (BaP), pyrene (Pyr), and phenanthrene (Phen), which differed in their physicochemical properties. The results showed that only 2 molecules, Phen and Pyr, were able to cross mammary cell layers. Phenanthrene radioactivity appeared more quickly in apical media, and its level after a 6-h exposure was 1.3 times higher than for Pyr and 7.7 times higher than for BaP. These findings suggested that mammary epithelium could play a key role in the selective transfer of PAH from food to milk.

(Key words: MAC T cell line, polycyclic aromatic hydrocarbon, mammary epithelium, transfer)

Abbreviation key: BaP = benzo[a]pyrene, PAH = polycyclic aromatic hydrocarbon, Phen = phenanthrene, Pyr = pyrene.

The hydrophobic character of polycyclic aromatic hydrocarbons (PAH) (n-octanol/water partition coefficients, log K_{ow} >4) and their resistance to biodegradation and elimination give them a tendency to accumulate in food chains (Feidt et al., 2000). Diet is one of the main sources of human and animal background exposure to these pollutants (Vyskocil et al., 2000). Several PAH are detected in milk and dairy products (Dennis et al., 1983; de Vos et al., 1990; Dennis et al., 1991; Grova et al., 2000).

Studies *in vivo* with rats prove that PAH are detected in the mammary gland after ingestion (Daniel et al., 1967). However, little is known about factors governing the transfer of these molecules through the mammary gland. Among PAH, the lightest and less lipophilic ones, such as naphthalene or phenanthrene (**Phen**), are detected in the greatest amounts in milk, whereas high molecular weight PAH are never detected (Dennis et al., 1983; Grova et al., 2000). Few *in vivo* studies on ¹⁴C-PAH transfer in ruminants corroborate these results (West and Horton, 1976; Eisele, 1985; Grova et al., 2002). These data support the hypothesis that PAH distribution in milk could be related to their physicochemical properties, and more particularly to their molecular weight and lipophilicity. However, no published experiment focuses on mammary epithelium role in these concentration differences.

In the present study, we used the MAC T cell line, established from bovine mammary cells transfected with SV 40 simian virus and obtained from Nexia Biotechnologies (Vaudreuil-Dorion, Canada), to study the uptake and transport of 3 ¹⁴C-labeled PAH—benzo[a]pyrene (**BaP**), pyrene (**Pyr**), and Phen—to determine the precise mechanism involved in their transfer through the mammary.

The cell line was maintained in complete Dulbecco's Modified Eagle's Medium containing 10% of fetal calf serum, penicillin (100 U/mL of medium), streptomycin (100 µg/mL), and gentamycin (100 µg/mL) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. Cells from passage 14 to 18 were seeded on filter inserts (4.2 cm², 1-µm pore size; Merck Eurolab, Fontenay sous Bois, France) at a cell density of about 225,000 cells/cm² and grown for 13 d. Medium was changed every 2 d.

Every contaminated culture medium contained 3.4 × 10⁻² µCi/mL [0.7 nM for 7,10-¹⁴C-BaP (Amersham, Buckinghamshire, UK) and 0.6 nM for 4,5,9,10-¹⁴C-Pyr (Sigma Aldrich, Saint Quentin Fallavier, France)] and 9-¹⁴C-Phen (Moravek Biochemicals, Brea, CA) dissolved in methanol (5 × 10⁻⁴%) and dimethylsulfoxide (0.1%) (Sigma Aldrich).

After 13 d of culture, the basal side received contaminated or control medium for 15, 90, 180, and 360 min.

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Four repetitions were achieved for each point, and 500 μL of basal and apical media were sampled in triplicate, added to 10 mL of Ultimagold (Packard, Rungis, France) scintillation liquid, and counted for 10 min using a Tricarb 460 CD liquid scintillation counter (Packard). Cells were rinsed with water, scraped, and collected in a solution of solune (Packard) and water (8:2). The mixture was kept at 50°C for 2 h and was finally counted in liquid scintillation with Hionic Fluor (Packard). Results were expressed in percentages of the radioactivity dose added to apical medium.

To characterize the transfer, a similar experiment was performed with a double concentration of ^{14}C -BaP and ^{14}C -Phe (6.8 $\mu\text{Ci/mL}$ corresponding to 1.4 nM for BaP and 1.2 nM for Pyr and Phen) to check the diffusion hypothesis.

Percentages of radioactivity counted were analyzed with the software STATBOX (Grimmer Software 2-5) by variance analysis in total randomization. The model included 2 factors: time (4 independent modalities: 15, 90, 180, and 360 min, with measures realized with different wells at each time) and molecule (3 independent modalities: BaP, Pyr, Phen), along with their interaction, time $\times$ molecule. Four repetitions were realized for each point, which meant that 4×3 molecules $\times$ 4 repetitions = 48 experimental units. The experiment with double concentration was analyzed using the same method.

After a 13-d culture, before and after a 3-h incubation with BaP, Pyr and Phen, histological studies showed that MAC T cells formed a differentiated confluent monolayer (data not shown). Moreover, transepithelial electric resistance measurement after incubations did not show important variations [300 Ω/cm^2 (SD = 30; $n = 96$)].

The levels of apical radioactivity measured through the permeable membrane without the cell monolayer reached about 14% for BaP, 25% for Pyr, and 27% for Phen as early as 15 min and remained quite stable all along the kinetics. The filter was then a barrier for the molecules, but further results confirmed that cells remained the principal determinant of their transfer.

About 1 and 0.1% of the total radioactivity added was recovered from the basal and apical walls cleaning after 6 h. These minor amounts would scarcely influence the kinetics.

Samples of apical and basal media and cells enabled an evaluation of the recovery rate for each PAH, which was constant for BaP, Pyr, and Phen (respectively 91.6, 90.2, and 86.6%).

For each molecule, radioactivity bound to cells (e.g., adsorbed to cell membranes or absorbed in cell cytoplasm) increased slowly until 180 min, when it reached between 0.3 to 0.5% of the radioactivity added basally

Figure 1. Part of radioactivity measured bound to MAC T cells between 15 and 360 min relative to the initial basal radioactivity. BaP = benzo[a]pyrene, Phen = phenanthrene, and Pyr = pyrene. Results are given as percentages of basally added radioactivity.

(Figure 1). Cell association profiles appeared to differ according to the molecule studied at 360 min ($P < 0.01$), reaching the highest level for Phen, followed by BaP and Pyr (1.1, 0.9, and 0.5%, respectively, of the dose added).

Transepithelial transfer rates were different for the 3 molecules (Figure 2). First, only 2 of the 3 studied micropollutants, Pyr and Phen, were able to cross the mammary barrier. Their radioactivity was readily observed after a 15-min incubation. Phenanthrene radioactivity was significantly greater, detected as early as 90 min ($P < 0.01$). It was vectorially discharged into the apical medium earlier (0.36% at 15 min) and continued to increase dramatically until 360 min, when 18.4% of the original basal dose appeared in the apical compartment. Pyrene showed a similar behavior. Its detected radioactivity significantly increased after 15 min and reached 13.8% at 360 min ($P < 0.01$). Release of radioactivity originating from BaP in the apical medium re-

Figure 2. Part of radioactivity measured in the apical medium between 15 and 360 min relative to the initial basal radioactivity and tendency curves. BaP = benzo[a]pyrene, Phen = phenanthrene, and Pyr = pyrene. Results are given as percentages of basally added radioactivity. Points with the same letter do not differ ($P > 0.05$).

Figure 3. a) Part of the radioactivity that was measured in the apical medium between 15 and 360 min after incubation with 3.4 (BaP) or 6.8 (BaP double) $\mu\text{Ci/mL}$ of ^{14}C -BaP in MAC T cells relative to the initial basal radioactivity. Results are given as percentages of basally added radioactivity. $n = 16$ (4 times, 2 concentrations, 2 repetitions). b) Part of the radioactivity that was measured in the apical medium between 15 and 360 min after incubation with 3.4 (Phen) or 6.8 (Phen double) $\mu\text{Ci/mL}$ of ^{14}C -Phe (b) in MAC T cells relative to the initial basal radioactivity. The Phen radioactivity transfer rate was independent from its initial basal concentration. Results are given as percentages of basally added radioactivity.

remained close to zero until 360 min, when it reached a very low level of 2.4%.

For each PAH, levels of radioactivity detected in the apical medium corresponded to a linear tendency curve, with a determination coefficient $R^2 = 0.86, 0.94$, and 0.97 , respectively, for BaP, Pyr, and Phen (Figure 2). Thus, radioactivity transfer rates would remain constant for the 3 compounds.

When ^{14}C -BaP and ^{14}C -Phen concentrations in the basal media were doubled, radioactivity bound to cells (data not shown) or detected in the apical medium did not differ significantly from results obtained previously (Figure 3a and b). The PAH radioactivity transfer rates were independent from their initial basal concentration, at least for those inferior to $6.8 \mu\text{Ci/mL}$ of culture medium concentrations.

The present investigation showed a transepithelial transfer of PAH. The quasi linear transfer in the apical compartment, an effective crossing in both senses (data not shown), and especially the maintenance of transfer rates after increasing media concentration, suggested a passive diffusion phenomenon. It may be argued that PAH transfer in mammary epithelium would be essentially linked to a phenomenon of diffusion.

Levels of radioactivity bound to cells remained quite low, but quantities were significantly different for the 3 compounds at 360 min. Transfer through mammary epithelium appeared significantly different for the 3 molecules. Pyrene and Phen appeared to be the compounds that were transferred the most rapidly and in the greatest amounts. After a 6-h exposure, Phen was transported into the basal side 1.3 and 7.7 times more than Pyr and BaP, respectively ($P < 0.01$). Studies in vivo with ruminants involving BaP, Pyr, and Phen also reported different distributions of the 3 PAH in milk after oral ingestion and corroborated our in vitro results (Table 1). In accordance with our results, the observations of Grova et al. (2002) showed close rates of transfer for Pyr and Phen (respectively, 1.9 and 1.5% of the ingested dose found in milk), even if the relationship was inversed.

Based on our knowledge concerning almost partial diffusion, it can be argued that physicochemical properties of PAH may play a key role in their transfer selectivity. Radioactivity quantities measured in basal medium were inversely related to their molecular weight (Figure 4), suggesting a phenomenon of intercellular diffusion. Apart from weight, it may also be argued that lipophilicity could explain the selective mammary transport too (Figure 4), suggesting a phenomenon of intracellular diffusion. However that may be, Phen, the

Table 1. Presence in milk and physicochemical properties of polycyclic aromatic hydrocarbons.

Molecule	Ring number	Molecular weight	Water solubility (mg/L)	$\log K_{ow}$	Presence in milk ($\mu\text{g/kg}$ of DM) (Dennis et al., 1983; Grova et al., 2000)
Phenanthrene	3	178.2	1.29 ± 0.07	4.6	9.5 to 12.5
Pyrene	4	202.3	1.30×10^{-1}	5.18	0.04 to 7.7
Benzo[a]pyrene	5	252	0.0038 ± 0.0003	6.5	<0.01

Figure 4. Relationship between the part of the organic micropollutants detected in the apical medium at 360 min and their physicochemical properties. Their molecular weight and their lipophilicity could explain the selective mammary transport of polycyclic aromatic hydrocarbons. Phenanthrene, the less lipophilic 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.

less lipophilic ($\log K_{ow} = 4.5$) and the lightest pollutant, would have the best transfer, whereas the other compounds would see their passage rate and quantity rate decrease as their lipophilicity and molecular weight increased. The selectivity of mammary epithelial barrier, notably according to the molecule lipophilicity and molecular weight, seemed to explain in part the reported differences in PAH concentrations detected in

milk or dairy products. However, it would be of great interest to determine the precise mechanisms involved using pharmacologic inhibitors or stimulators of transport.

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