The effect of pre-exposition to PCB 126 on metabolism and nucleotide adduct formation of benzo(*a*)pyrene in the fish hepatoma cell line PLHC-1.

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Abstract

In this study it was shown that, in the fish hepatoma cell line PLHC-1, CYP1A induction by dioxinlike compounds, e.g. PCB 126, can increase the metabolism and RNA adduct formation of benzo(*a*)pyrene significantly.

Introduction

Fish are exposed to a wide variety of contaminants, often including polynuclear aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). In some contaminated areas an elevated frequency of liver neoplasia has been observed in bottom dwelling fish in the past [1,2]. At these sites levels of PAHs were typically several orders of magnitude greater than those at less polluted sites. In addition, extracts of sediments that were highly contaminated with PAHs could elicit liver tumours in rainbow trout [3]. Furthermore, it was shown that at least two PAHs: benzo(*a*)pyrene (BaP) [4] and 7,12 dimethyl-benzanthracene[5] cause liver neoplasia in fish. These carcinogenic PAHs have to be activated by oxidative metabolism, mainly by cytochrome P-450 monooxygenases and flavoprotein oxygenases. BaP is often used as a model compound to investigate this bioactivation process. It binds to DNA and other cellular macromolecules efficiently, because of its reactive bay region epoxide group[6,7,8]. Several Cytochrome P450 enzymes are involved in the oxidation of BaP. *In vivo* and *in vitro* experiments with mammalian and fish species produced evidence that cytochrome P4501A(1) is important for this bioactivation of BaP[6,9]. In many vertebrate species CYP1A(1) is highly inducible by PAHs and polyhalogenated aromatic hydrocarbons (PHAHs), including PCBs. In this study, the question of the relevance of CYP1A(1) induction by e.g. dioxinlike compounds for PAH bioactivation was investigated in PLHC-1 hepatoma cells, derived from a topminnow species (*Poeciliopsis lucida*)[10]. BaP was used as a carcinogenic PAH and 3,3'4,4'5 pentachloro-biphenyl (PCB 126) because it causes CYP1A (1) induction in most species, including fish [11]. Both compounds are commonly found in the aquatic environment and fish are often simultaneously exposed to both types of compounds, each having a distinctly different mode of action.

Experimental Methods

Chemicals. TRIzol reagent, DMEM and medium supplements were obtained from Gibco BRL, Life technologies (gaitersburgh, MD, USA), except foetal calf serum (Flow, ICN Biomedicals, Amsterdam, the Netherlands). BaP (98 %) originated from Sigma Chemical Co. (St Louis, Mo, USA); ^{3}H-BaP (55 Ci/mmol) from Amersham Int. plc (Little Chalfont, England). PCB 126 (purity > 99 %) from Schmidt BV (Amsterdam, the Netherlands.).

Cell culture conditions. PLHC-1 Cells were grown in 9 cm culture dishes, at 30° C, under 5 % CO_2, in Dulbecco's Modified Eagle's Medium (D-MEM) with 10 % foetal calf serum added [12,13]. Plates were seeded with 3.10^6 cells after reaching 100 % confluency ($\cong 30.10^6$ cells/dish) after 6 - 7 days. BaP and PCB 126 were added to the dishes dissolved in dimethyl sulphoxide (DMSO, max. 0.3 %). Controls received DMSO only. Cells were exposed to PCB 126 for 24 hours; ^{3}H-BaP: 4 hours, unless indicated otherwise.

Enzyme and protein assays. Ethoxyresorufin-*O*-deethylase activity (EROD) was used as marker for CYP1A(1) activity. EROD activities were measured on a Cytofluor 2300 fluorescence plate reader (Millipore), using a method adapted from Hahn *et al.*[14].
GST activities were measured using 1 chloro-2,4-dinitrobenzene (CDNB) as a substrate, and determined in cytosol, according to the method of Habig *et al.* [15]. Protein concentrations of cell-suspensions and cytosols were measured according to Bradford [16].

BaP metabolism and DNA/RNA adducts. A stock solution of ^{3}H-BaP was purified according to the method of Van Cantfort *et al.* [17]. BaP metabolism was determined by using a modified extraction method described by Cantfort *et al.* [17] and Egaas *et al.*[18].
DNA and RNA adducts were determined by isolating RNA and DNA and measuring the radioactivity bound to it. The RNA/DNA isolation was performed using TRIzol reagent, and was based on the single-step RNA isolation developed by Chomczynski *et al.*[19].

Results

CYP1A induction by PCB 126.
Maximum EROD induction was reached after 24 hours of dosage and maintained until 72 hours. Maximum activity (120 pmol resorufin/mg protein . min.) was reached at 1 nM and the EC_{50} was 0.2 nM. At higher concentrations a decrease in EROD activity was observed.

GST activity following exposition to PCB 126 or BaP
GST activity towards CDNB was measured after exposure of PLHC-1 to an extensive range of BaP (0.1 nM - 10 µM) or PCB 126 (1 pM - 100 nM) concentrations. No effect on GST activity was observed for both compounds with basal activity remaining at 1.5 ± 0.2 nmol/minute. mg protein

Time dependent CYP1A induction, BaP metabolism and adduct formation
EROD activity reached a maximum (≥ 55 pmol/mg.min) between 4 and 6 hours after the start of the exposition and declined afterwards. 4 and 12 hours after exposure respectively 57.7 ± 0.2 % and 88 ± 0.7 % of 100nM BaP was metabolised. The formation of RNA adducts was almost linear in time for 24 hours, at a rate of 96 x10^6 adducts/µg RNA per hour (R^2=0.988), but the number of DNA adducts decreased after 12 hours incubation.

Dose-response for BaP and metabolism, adduct formation and CYP1A induction.
Maximum EROD activity was reached at a concentration of approximately 100nM BaP and the EC_{50} was approximately 5nM. In the concentration range from 1 to 300 nM BaP the percentage of metabolised BaP decreased only slightly (from 56.1 ± 0.4 to 50 ± 2 %), but at higher concentrations a significantly smaller proportion is metabolised. It was observed that with a decrease in the percentage of BaP that is metabolised, the percentage of primary metabolites increased. An increase was observed for both DNA and RNA-adducts with an increasing BaP concentration, when measured in absolute numbers. If normalised on the metabolized amount of BaP only, a slight increase (approx. 2-fold) in DNA adducts from 3 to 3000 nM BaP was noticed.

Influence of PCB 126 pre-treatment on BaP metabolism, RNA adduct formation and CYP1A induction.
Based on the earlier dose-response experiment with PCB 126, three concentrations of PCB 126 were chosen representing the LOEL, the EC_{50},and the EC_{100} of the EROD activity. PLHC-1 cells were pre-incubated with these PCB 126 concentrations and then received a dose of BaP at three different concentrations. The metabolism of BaP remained remarkably constant at 56 ± 4 % in spite of the pre-induction with PCB 126. Only at the highest concentration of BaP, 1000nM, a significant dose dependent increase in metabolism from 35 ± 2.6 to 53 ± 7 % was observed (Figure 1).

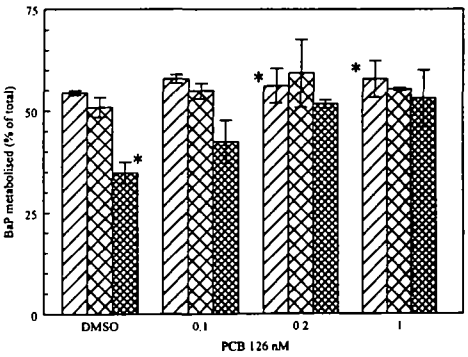

Figure 1 Influence of pre-treatment with three concentrations of PCB 126 on the metabolism of BaP in PLHC-1. 10nM BaP: ▨ ; 100nM: ▩ ; 1000nM: ▩ . Error bar indicates sd of 3 values (except: *: range of 2).

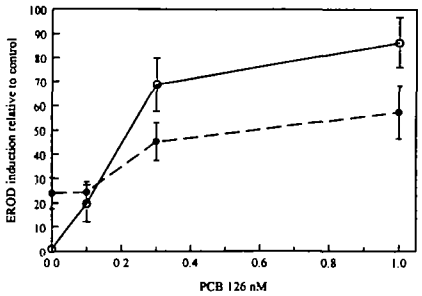

Figure 2: Induction of EROD activity after treatment by PCB only (—o—) or by PCB + 100nM BaP (—●—). Error bar indicates sd of 3 values

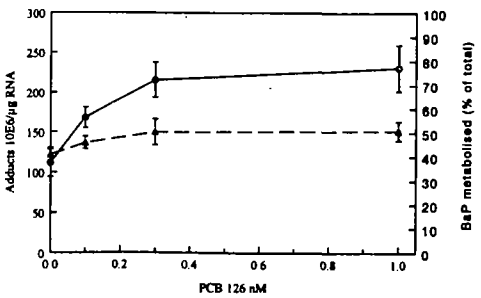

figure 3: RNA adducts (—o—) and BaP metabolism (—▲—) after 4 hours exposition to 100 nM BaP. PLHC-1 cells were pre-treated with three concentrations of PCB 126 or DMSO. Error bar indicates sd of 3 values.

In a second experiment only 100nM BaP was used. EROD induction by BaP alone was 24 fold. At the two highest PCB concentrations (0.3 and 1.0 nM) pre-treatment with PCB resulted in higher EROD induction relative to BaP alone, but lower induction relative to the PCB alone (figure 2). In figure 3 it can be observed that there is a moderate increase in metabolism from 41 ± 3 to 51 ± 4 % of BaP, when compared with cells treated to DMSO or 1 nM PCB 126. This increase in BaP metabolism concurs with a significant increase in RNA adduct formation (approximately 2-fold).

Discussion

Our EROD results for PCB 126 are in good agreement with those observed by Hahn *et al.*[20] who reported an EC_{50} of 0.38nM and maximum activity at 3nM. Our values were respectively 0.2 and 1nM. A distinct difference was observed between both compounds in the time course of CYP1A induction. Rapid metabolism of BaP compared to PCB 126 could be the major contributing factor to this difference. Our data show that care should be taken when comparing dose-response curves and EC_{50}'s for kinetically distinctly different compounds like BaP and PCB 126.

BaP, like PCB 126, causes a decline in EROD induction at higher concentrations. This biphasic response of CYP1A activity has also been reported from other *in vitro* studies [19,20]. This phenomenon could be caused by inhibition or inactivation of the CYP1A protein by BaP or PCB. It has also been suggested that EROD inhibition may be linked to deregulation of the heme biosynthesis [14,21,22,23].

In the experiments described in this paper, intact cells were used instead of subcellular fractions, which is a step closer to the in vivo situation. Pre-incubation of PLHC-1 cells with PCB 126, causing up to 80-fold induction of EROD (CYP1A) activity, did not enhance metabolism of BaP, except at a very high BaP concentration (1 μM). These results suggest that, in the fish cell line PLHC-1, CYP1A is only of minor importance for the phase I metabolism of BaP at concentrations ≤ 100 nM.

Both RNA and DNA adducts of BaP were measured to determine the formation of reactive BaP metabolites. RNA adduct levels were up to 5 times higher than DNA adduct levels.

The dose-response relationships of EROD induction and RNA adduct formation by BaP in the PLHC fish cells show a remarkable concurrence, suggesting a link between the two mechanisms. However, it cannot be discerned if this concurrence is a result of increased BaP concentrations or a direct effect of CYP1A induction.

The combination experiment (figures 1,2 and 3) investigated the possible relationship between CYP1A induction and BaP activation. In the PLHC cells a pre-exposition to 1 nM PCB 126 lead to a 20 % increase in metabolism of BaP, while RNA adduct formation was more than 100 % induced. This suggests that CYP1A is specifically involved in the activation of BaP. These results are in agreement with other studies, using human and rat hepatocytes, which showed a moderate increase in DNA-adduct formation after pre-exposure to CYP1A inducers [24,25].

In our experiments the magnitude of CYP1A induction is much greater than the increase of BaP adduct formation. We speculate that phase II metabolism, e.g. GST activity, may be an important reducing factor in the formation of BaP adducts to cellular macromolecules. However, in our PCB 126 and BaP combination study GST activity remained unchanged over the whole concentration range of both compounds.

Based on our results obtained in the PLHC fish cell line it can be concluded, that CYP1A induction by dioxinlike compounds, e.g. PCB 126, can increase the metabolism of BaP but

also the activation of BaP to reactive intermediates significantly. As many bottom dwelling fish species, e.g. flounder and dab, are exposed directly to both type of compounds an interaction between both mechanisms of action might occur, possibly resulting in an increase in liver neoplasia. However, any predictions from our results towards the *in vivo* situation are at present impossible due to the lack of knowledge about differences in species sensitivity.

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Literature cited

1)Malins, D.C., M.M. Krahn, M.S. Myers, L.D. Rhodes, D.W. Brown, C.A Krone B.B. McCain, S.L. Chan, 1985. Carcinogenesis 6, 1463-1469.
2) Malins, D.C., B.B. McCain, D.V. Brown, S.L. Chan, M.S. Myers, J.T. Landahl, P.G. Prohaska, A.J. Friedman, L.D. Rhodes, D.G. Burrows, W.D. Gronlund, H.O. Hodgins, 1984. Environ. Sci Technol. 18, 705-713.
3) Metcalfe, C.D., V.W. Cairns, J.D. Fitzsimons, 1988. J. Fish. Aquat. Sci. 45, 2161-2167.
4) Hendricks, J.D., T.R. Meyers, D.W. Shelton, J.L. Casteel, G.S. Bailey, 1985. J. Natl. Cancer Inst. 74, 839-851.
5) Schultz, M.E. and R.J. Schultz, 1982. Environmental research 27, 337-351.
6) Varanasi U. Ed., 1989. Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. Boca Raton: CRC press.
7) Dipple, A., R.C. Moschel, C.A.H. Bigger, 1984. Polynuclear aromatic carcinogens in Chemical Carcinogens Vol. 1, 2nd ed. C.E. Searle Ed., American Chemical Society, Washington DC.
8) Thakker, D.R., H. Yagi, W. Levin, A.W. Wood, A.H. Conney, D.M. Jerina, 1985. Polycyclic aromatic hydrocarbons: metabolic activation to ultimate Carcinogens. in Bioactivation of foreign compounds, Anders M.W. Ed. New York, Academic Press.
9) Stegeman, J.J. and J.J. Lech, 1991. Environ. Health Perspect. 90, 101-109.
10) Hightower, L.E. and J.L. Renfro, 1988. J. Exp. Zool. 248, 290-302.
11) Safe, S., 1990. Crit. Rev. Tox. 21(1), 51-88.
12) Tillitt, D.E., 1993. Report from the Uppsala workshop on reproduction disturbances in fish. p. 97-101.
13) Tillitt, D.E., J.P. Giesy, G.T. Ankley, 1991. Environ. sci. technol. 25, 87-92.
14) Hahn, M.E., T.M. Lamb, M.E. Schultz, R.M. Smolowitz, J.J. Stegeman, 1993. Aquat. Tox. 26, 185-208.
15)Habich, W.H., M.J. Pabst, W.B. Jakoby, 1974. J. biological Chem. 249(22), 7130-7139.
16) Bradford M.M., 1976. Anal. Biochem. 72, 248-254.
17)Cantfort van J., J. De Graeve, J.E. Gielen, 1977. Biochem. Biophys. Res. Comm. 79(2), 505-512.
18) Egaas, E. and U. Varanasi, 1982. Biochem. Pharmacol. 31, 561-566.
19) Chomczynski, P., N. Sacchi, 1987. Anal. Biochem. 162, 156-160.
20) Hahn, M.E. and K. Chandran, 1996(2). Arch. Bioch. Biophys. 329(2), 163-174.
21) Gooch J.W., A.E. Elskus, P.J. Kloepper-Sams, M.E. Hahn, J.J. Stegeman, 1989. Tox. Appl. Pharm. 98, 422-433.
22) Hahn, M.E., B.L. Woodward, J.J. Stegeman, S.W. Kennedy, 1996(1). Env. Tox. Chem. 15(4), 582-591.
23) Tysklind, M., A.T.C. Bosveld, P. Andersson, E. Verhallen, T. Sinnige, W. Seinen, C. Rappe, M. van den Berg, 1995. Env. Sci. & Pollut. Res. 2(4), 211-216.
24) Monteith, D.K., D. Ding, Y.T. Chen, G. Michalopoulos, S.C. Strom, 1990. Tox. Appl. Pharm. 105, 460-471.
25) Eberhart, J., S.L. Coffing, J.N. Anderson, C. Marcus, T.J. Kalogeris, W.M. Baird, S.N. Park, H.V. Gelboin, 1992. Carcinogenesis 13(2), 297-301