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Atrazine and PCB 153 and their effects on the proteome of subcellular fractions of human MCF-7 cells

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ABSTRACT

Several man-made organic pollutants including polychlorinated biphenyls (PCBs) and several pesticides may exhibit endocrine disrupting (ED) properties. These ED molecules can be comparatively persistent in the environment, and have shown to perturb hormonal activity and several physiological functions. The objective of this investigation was to study the impact of PCB 153 and atrazine on human MCF-7 cells, and to search for marker proteins of their exposure. Cells were exposed to environmentally high but relevant concentrations of atrazine (200 ppb), PCB 153 (500 ppb), 17-β estradiol (positive control, 10 nM) and DMSO (0.1%, negative control) for t = 36 h (n = 3 replicates/exposure group). Proteins from cell membrane and cytosol were isolated, and studied by 2D-DiGE. Differentially regulated proteins were trypsin-digested and identified by MALDI-ToF-ToF and NCBInr database. A total of 36 differentially regulated proteins (>|1.5| fold change, $P<0.05$) were identified in the membrane fraction and 22 in the cytosol, and were mainly involved in cell structure and in stress response, but also in xenobiotic metabolism. 67% (membrane) and 50% (cytosol) of differentially regulated proteins were more abundant following atrazine exposure whereas nearly 100% (membrane) and 45% (cytosol) were less abundant following PCB 153 exposure. Western blots of selected proteins (HSBP1, FKBP4, STMN1) confirmed 2D-DiGE results. This study emphasizes the numerous potential effects that ED compounds could have on exposed humans.

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1. Introduction

Endocrine disrupting compounds (EDCs) are molecules exhibiting similarities to human hormones that may modulate their activity and perturb normal physiological functions. Both natural compounds, e.g. certain mycotoxins and man-made pollutants can act as EDCs. To reduce human exposure, efforts have been made to avoid environmental contamination by these compounds. Prominent examples include polychlorinated biphenyls (PCBs), which have been produced on industrial scale since the 1920s and were used in dyes, condensers, or in transformers, but which were banned in the 1980s. PCBs can be differentiated into dioxin-like species and non-dioxin like PCBs. The former are generally more toxic due to their binding to the aryl hydrogen receptor and their higher carcinogenicity [1]. However, the long persistence of PCBs in the environment and the food chain, especially in lipid-soluble tissue such as in eels [2,3], polar bears [4], and seals [5], but also in human blood [6,7] represents a continuing problem, and concentrations of up to several 100 ppb may still be found in animals ranking high in the food chain, such as in fish [3]. In recent years, several PCBs or their hydroxylated metabolites have been reported to possess estrogenic effects [7,8], leading to defective reproduction in animals [9,10] and possibly in humans [11,12], perturbed immune functions [13], cancer [14], adrenal effects [15] and abnormal thyroid function [16] in culture cells and animal trials.

In addition to PCBs as industrial pollutants, other endocrine disrupting compounds have been employed in e.g. agriculture, including several pesticides, such as the banned insecticide DDT, while others are still in use, including the triazine-herbicides such as terbutylazine, cyanazine, simazine and atrazine, with the latter still constituting the most used herbicide in many countries such as the US, and possibly worldwide [17]. In Europe, usage of atrazine was banned in 2004; however, significant concentrations can still be found in the ground [18] and surface water [19,20]. Even though the strict classification of atrazine as an EDC has been debated [21], more and more evidence for this is accumulating [22]. It has been suggested to affect the central nervous system [23,24], the endocrine system [25–27] and the immune system [28,29] and reproductive health of rats [30,31], pigs [32], and amphibians [26,33]. Even low level short term exposure to atrazine increased the number of intersex frogs [34], feminization

Abbreviations: PCBs, polychlorinated biphenyls; ATR, atrazine; US-EPA, US Environmental Protection Agency; E2, estradiol; ROS, reactive oxygen species; 2D-DiGE, 2D difference in gel electrophoresis; EDC, endocrine disrupting compounds

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[35], and aberrant gonadal development [26,33], potentially due to its action on aromatase [36]. However, their impact on mammals and particularly on humans is controversial, due to a lack of data.

Thus, alternative methods to assess the impact of EDCs on humans are needed. Apart from a previous study of ours [37], only one publication investigated the effects of atrazine at the proteome level, using LbetaT2 rat pituitary cells [38], however, another recent publication showed effects of PCB exposure on the tadpole proteome [39]. The objective of the present investigation was to better understand the effects of PCB 153 and atrazine on subcellular compartments of human MCF-7 cells. For this purpose, MCF-7 cells were exposed for 36 h to 200 and 500 ppb of atrazine and PCB 153, respectively, together with a hormone stimulated control (17-beta-estradiol) and an untreated control (DMSO), and the proteome of the cellular membranes and the cytosol were studied independently.

2. Materials and methods

2.1. Chemicals and materials

2.1.1. Atrazine

Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, Lyon, France) and were of analytical grade or superior. Atrazine (98% purity) was purchased from Chemservice (West Chester, PA). Atrazine was dissolved in 100% DMSO at a concentration of 10 mg/mL. A working solution (0.1 mg/mL atrazine) was made by dilution of the stock solution in DMSO. This working solution was added directly to the cell culture medium (dilution 1/500) to obtain the final 200 μg/L concentration (= 200 ppb).

PCB 153 (98% purity) was dissolved in 100% DMSO to prepare a 2 mg/mL stock solution. As for atrazine, a 0.1 mg/mL working solution was prepared for PCB 153 by dilution in DMSO and was added to the culture medium to obtain the 500 μg/L concentration. PCB 153 was chosen due to its wide abundance and belonging to one of the indicator PCBs for industrial pollution, even though not possessing any dioxin-like properties such as binding to the aryl hydrocarbon receptor (AhR) [14,40]. 17-β estradiol was dissolved in DMSO to obtain a 10 μM working solution which was directly added to the culture medium to obtain a final 10 nM concentration in the estrogen stimulated positive control. Control cultures were kept in 0.1% DMSO and are as negative control.

2.2. Cell cultures

Estradiol responsive MCF-7/BUS cells (ER^+ human mammary carcinoma cells expressing estradiol receptors, as described previously [41]) were grown as monolayers in TC175 flasks at 37 °C in a humidified atmosphere of 5% CO_2 in air in order to obtain a high cell density without reaching confluence. This was achieved by seeding 12 million cells in a TC 175 cm flask and two days of growth in MEM (Minimum Essential Medium Modified, Invitrogen SARL, Cergy Pontoise, France), supplemented with 10% fetal calf serum (FCS), 0.22% sodium bicarbonate (Invitrogen SARL), penicillin and streptomycin (40 IU/mL, Invitrogen SARL), and 0.2 M HEPES. Cells were then transferred for 24 h to a medium containing charcoal depleted bovine serum (CD-FBS) without phenol red, prior to treatment with either 0.1% DMSO, 10 nM 17-β estradiol, 200 ppb atrazine or 500 ppb PCB 153 (final concentrations in culture medium) in the same CD-FBS. Thus, roughly 30 million cells per TC175 were exposed under different conditions for 36 h before harvesting.

2.3. Protein extraction from membrane and cytosol fraction and analysis

Three TC175 flasks of each treatment (3 to 7×10^7 cells) were used to obtain sufficient biological material. Cells were extensively washed with ice cold phosphate buffered saline, rinsed with a solution at pH 7.5 containing 100 mM PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)), 70 mM NaCl and protease inhibitor, and then detached by scrapping. Cells were collected and transferred in a French Press Disrupter (Thermal Electron Corporation, Waltham, MS) at a pressure of about 150 psi, resulting in disruption of cellular membranes without affecting nuclei and mitochondria. The cell lysate was checked by Trypan blue staining to verify the disruption of cell membranes and then nuclei were sedimented by centrifugation for 10 min at 1000 g (Beckmann Avanti™ 30 Centrifuge, Beckmann Coulter, Palo Alto, CA) at 4 °C. The resulting pellet contained intact nuclei, while the supernatant contained the cytosolic fraction, mitochondria and cellular membranes. The pellet was discarded and the supernatant was centrifuged at 20,000 g for 25 min at 4 °C in order to remove mitochondria and nucleus fragments. The supernatant was centrifuged at 90,000 g for 90 min at 4 °C to pellet membranes and to remove the majority of the cytosolic fraction, which was further processed as described below.

The pellet containing membrane proteins was resuspended in 250 μL of NH_4HCO_3 100 mM and 3 mL of Na_2CO_3 100 mM at pH 11.4, both containing protease inhibitors, and then centrifuged at 90,000 g, for 45 min at 4 °C. The pellet was resuspended again in 250 μL of NH_4HCO_3 100 mM and then 3 mL of KCl 1 M, both containing protease inhibitors, and then centrifuged at 90,000 g for 45 min at 4 °C. The pellet of membrane proteins was resuspended in 1 mL NH_4HCO_3 100 mM containing protease inhibitors and precipitated by centrifugation at 90,000 g for 90 min at 4 °C. Finally, the pellet of membranes was resuspended in 50 μL of a solution containing NH_4HCO_3 25 mM, SDS 2%, Na-deoxycholate 2% and protease inhibitors and then stored at −20 °C until analysis.

To the cytosolic fraction, 4 volumes of ice-cold acetone/TCA (80%/20%) were added, stored overnight at −20 °C and then proteins were precipitated by centrifugation at 10,000 g (Allegra 64 R centrifuge, Beckmann Coulter) for 10 min at 4 °C. The protein pellet was then dried for 5–10 min under vacuum and dissolved in labeling buffer containing 30 mM Tris, 2 M thiourea, 7 M urea, 4% w/v CHAPS by pipetting and centrifugation at 15,000 g at 15 °C during 10 min. After spermidine addition to 1 mM final concentration, the samples were centrifuged at 15 °C and 26,000 g for 2 h. These samples were used immediately or stored at −20 °C until further analyses.

The protein concentration in these fractions was determined using a quantification kit (2D Quant Kit, Amersham Biosciences, Uppsala, Sweden) and BSA (2 mg/mL) as a standard, following the manufacturer's instructions. After extraction, proteins were used for a multiplexed analysis by 2D-DiGE as described by Skynner et al. [42].

2.4. Protein labeling

Unless otherwise mentioned, all equipment was obtained from GE Healthcare (Uppsala, Sweden). The pooled “internal standard” added to each gel-run was prepared with 1/12 of each protein extract. Protein extracts and the pooled internal standard were labeled prior to mixing and electrophoresis with the CyDyes™. 12 gels (3 biological replicates for each condition (DMSO, 17-β estradiol, atrazine, PCB 153) run in duplicate to allow for dye swapping) were run for the cytosol and membrane proteins, respectively, each containing internal standard, and 2 protein extracts obtained from two exposure treatments (Table 1). Each protein extract was thus labeled at a minimum ratio of 30 μg protein: 240 pmol Cy2, Cy3 or Cy5 protein labeling dye, vortexed, and incubated on ice for 30 min in the dark as described previously by Skynner et al. [42].

The reactions were quenched by addition of 1 μL of 10 mM lysine, vortexed, and incubated on ice for 10 min in the dark. An equal volume of “2× lysis” buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% w/v DTT) was added. The samples were vortexed and incubated on ice for a further 15 min in the dark. Then, the pooled Cy2-labeled internal standard was combined with the Cy3-labeled and Cy5-labeled

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