EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON GLOBAL DNA METHYLATION AND DIFFERENTIATION USING *IN VITRO* MODELS

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Introduction

In recent decades the incidence of obesity has grown to epidemic proportions globally. Although obesity is considered a disorder of energy imbalance imposed on a background of genetic pre-disposition, there is much uncertainty about the aetiology and molecular mechanisms underlying this condition[1]. Evidence is accumulating that factors in early development such as exposure to environmental contaminants may contribute to the development of obesity later in life[2, 3]. Epidemiological studies have uncovered associations between *in utero* exposure to EDCs and the development of obesity later in life[4]. Animal studies also indicate that *in utero* and neonatal exposure to environmental chemicals may lead to weight gain in later life[5]. *In vitro* studies have linked exposure to environmental chemicals to enhanced adipogenesis[6], which may be a contributing factor to increased body fat mass and the onset of obesity.

A possible explanation for the developmental origins of obesity and related disorders may be found in epigenetics. Epigenetic modifications play a role in the control of gene expression by regulating the accessibility of chromatin for the transcriptional machinery without altering the DNA sequence itself. The most well studied epigenetic mechanism is DNA methylation. Abnormal epigenetic programming through DNA methylation can lead to aberrant genomic responses and ultimately altered cell function. Support for the rationale that epigenetic alterations caused by chemical exposure during early development may lead to increased susceptibility to disease comes from animal studies[7].

Given the evidence that chemicals may play a role in the developmental programming of obesity, and that altered epigenetics may form an underlying mechanism, the aim of this study was to examine the effects of a range of endocrine disrupting chemicals (EDCs) on genome-wide DNA methylation. To this end, *in vitro* models were selected which represent two important tissues in the regulation of energy metabolism and obesity, namely the hypothalamus and adipose tissue[8]. In addition, the effect of these chemicals on the differentiation of 3T3-L1 pre-adipocytes to adipocytes was investigated.

Materials and methods

Murine and human neuronal cells (N2A and SK-N-AS) and murine embryonic pre-adipocyte fibroblasts (3T3-L1) were exposed to compounds from five major classes of environmentally occurring EDCs shown previously in either human or animals to be implicated in obesity and related disorders[9]. These compounds included tetra-chloro-dibenzo-[*p*]-dioxin (TCDD) representing the group of dioxin-like polyhalogenated hydrocarbons (PHAHs); 2,2',4,4',5,5'-hexachlorobiphenyl PCB-153 representing non dioxin-like PHAHs; HCB representing organochlorine (OC) pesticides; hexabromocyclododecane (HBCD) and 2,2',4,4'-tetrabrominated diphenyl ether (PBDE-47) representing brominated flame retardants (BFRs) and perfluorinated octyl acid (PFOA) and perfluorinated octyl sulfonate (PFOS) representing perfluorinated compounds (PFCs). In addition, tributyltin chloride (TBT), diethylstilbestrol (DES) and bisphenol A (BPA) were included in the study.

N2A and SK-N-AS cells were seeded in 6-well plates at a density of 50000 cells/well. At 24 hrs after seeding, cells were exposed to 0.1 μM TBT or TCDD, 1 μM 5-AC or HCB, or 10 μM BPA, DES, PFOA, PFOS, HBCD, PCB153, or BDE-47 for 48 hrs (N2A) or 72 hrs (SK-N-AS). 3T3-L1 cells in passage 12 were seeded in 6-wells plates at a density of 150000 cells/well. Two days after confluence, differentiation was induced with culture medium supplemented with 1 μM dexamethasone, 1.67 μM insulin and 5 mM 3-isobutyl-1-methylxanthine (IBMX) as previously described[10] and refreshed every day. Exposure to 0.1 nM TCDD, 10 nM TBT, 1 μM HCB or troglitazone (TRO), 2.5 μM BDE-47, 3.4 μM PCB153, or 10 μM BPA, DES, PFOA, PFOS, or HBCD was

started at the moment of differentiation induction. Three days after starting the induction, cells were refreshed with full culture medium which included the test compounds and 1.67 µM insulin. Cells were kept in these conditions with medium refreshed every other day until day 8. Global methylation was investigated using HPLC. After exposure, DNA was extracted from cells and stored at -20ºC until further analysis. Prior to DNA purification, the lysates were treated with 1 mg/ml RNAse A for 5 minutes at room temperature. For analysis of global methylation, DNA samples (1 µg) were digested to single nucleotides with a combination of DNAse I and Nuclease P1. Samples were further digested with alkaline phosphatase, and deoxycytidine (dC) and 5-methyl deoxycytidine (5MdC) were quantified with an HPLC-UV system (Shimadzu) equipped with a 125x4mm Nucleosil 100-10 SA column and a mobile phase consisting of 40 mM acetic acid in 15% acetonitrile (pH 4.8) at a flow rate of 0.6 ml/min. Percentage methylation was calculated with [5MdC]/ ([5MdC] + [dC])*100[11]. Differentiation was assessed by fluorescence-activated cell sorting (FACS). Cultured cells were tripsinized and resuspensed in PBS. Paraformaldehyde was added to a final concentration of 0.5%. Samples were kept on ice and centrifuged for 3 min at 100 x g. Paraformaldehyde solution was removed and Nile Red dissolved in FACS buffer was added to a final concentration of 0.025 µg/ml. Nile Red staining and granularity were quantified by measuring 10.000 cells at a wavelength of 530nm on a FACSCalibur Dual-Laser Benchtop Flow Cytometer[12]. Data was acquired and analyzed with Flowjo software. Univariate treatment effects were determined by one-way ANOVA analysis ($p<0.05$) on data that were normally distributed by themselves or after ln-transformation (Kolmogornov-Smirnov test). Significant differences from the vehicle control treatment were determined by Dunnet's posthoc multiple comparison test for data with equal variances (Levene's test) or by Dunnett's T3 test for data with unequal variances. All univariate analyses were performed using the SPSS Software Package (Version 16.0, 2007).

Results and discussion

Global DNA methylation was determined in a human (SK-N-AS) and a murine neuronal cell line (N2A) exposed to non-cytotoxic concentrations of EDCs. Exposure of both cell lines to DNA methyltransferase-1 (DNMT1) inhibitor 5-AC resulted in a 30% reduction of global DNA methylation in SK-N-AS cells and 20% reduction in N2A cells. Exposure to the selected EDCs, however, did not result in any effects on global DNA methylation (data not shown).

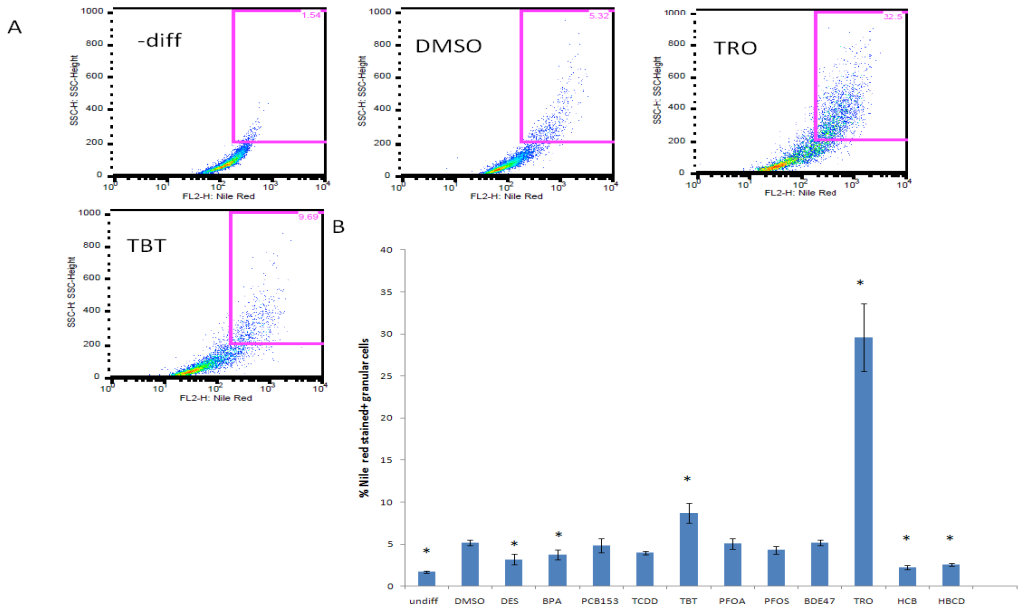

Fig.1. Differentiation to adipocytes after exposure to EDCs. (A) Plots of granularity versus Nile Red fluorescence and (B) histogram of percentage adipocytes.

Differentiated 3T3-L1 cells in passage 12 were analyzed for two characteristics of lipid accumulation, i.e. Nile Red fluorescent lipid staining and surface granularity due to lipid droplets in the cytoplasm. Plots of granularity versus Nile Red fluorescence showed an increase in cells defined as adipocytes in the selected gap (Fig. 1A, pink square) with average values of 30% for TRO and 9% for TBT compared to 5% for DMSO controls (Fig. 1B), suggesting enhanced differentiation by TBT. Exposure to DES, BPA, HCB and HBCD led to a significant decrease in differentiation when compared to DMSO control. For PFOA, PFOS, BDE-47, PCB-153, and TCDD no significant changes in differentiation were found (Fig. 1B).

Effects of selected EDCs on global methylation in adipose cells were investigated in 3T3-L1 cells which had undergone differentiation according to the same regime used for analyzing the effects of EDCs on adipogenesis (Fig. 1). A significant decrease in DNA global methylation status was found in differentiated DMSO control cells (4.04%) compared to undifferentiated cells (4.15%) (Fig. 2). A decrease in global methylation status compared to the control cells was found for cells treated with TRO (3.85%) and TBT (3.94%). Exposure to the selected EDCs (DES, HCB, PCB-153 and PFOA) did not show any effects on global methylation status when compared to control (Fig. 2). These results show that, during differentiation, a process of demethylation may take place and that exposure to compounds as TBT may induce further demethylation. HCB, PCB-153 and PFOA were unable to alter methylation in the conditions tested.

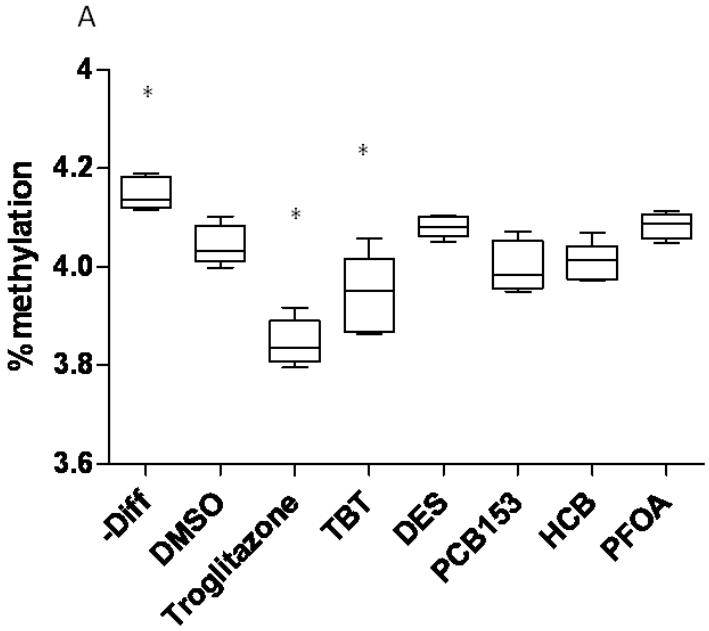

Fig.2. Global methylation in 3T3-L1 cells.

TBT was found to be an inducer of adipogenesis as suggested by Grün *et al.*[13]. TBT is considered a ligand for the retinoid X receptor and the peroxisome proliferator-activated receptor (PPAR) γ[13]. Its positive effect on adipogenesis may be explained by its PPARγ agonistic properties[13, 14]. All other compounds tested here have been associated with obesity or obesity-related endpoints in epidemiological or animal studies[9]. However, the mechanism underlying this association may not be directly related to increased adipogenesis. It is known that the response to EDCs depends on timing, dose and gender [15]. In the doses tested here, these EDCs did not directly enhance adipogenesis. This may be due to the concentrations tested, as for the compound BPA, exposure to a higher concentration has resulted in induced adipogenesis in 3T3-L1 cells[16]. However, in other cases, it may be that adipogenesis does not play a role in their obesogenic activity, and other mechanisms such as alterations in thyroid and steroid hormones should be researched.

This study also shows that TBT affects global methylation in an *in vitro* model for adipocyte differentiation. This result is important in the light of previously reported effects of prenatal TBT exposure in mice on adult onset of obesity [13.] DNA hypomethylation induced by TBT has also been suggested by Wang *et al*[17] in the liver of *Sebasticus marmoratus* fish. However, we did not find effects of other EDCs on global methylation using these *in vitro* models. It is possible that these EDCs do not influence global DNA methylation, or that the selected *in vitro* models may not be appropriate to detect these changes. It should also be kept in mind that this global analysis may not detect more subtle effects such as alterations of DNA methylation on a small number of CpGs in target promoters. Further epigenetic analysis of specific target genes is therefore recommended.

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