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Transcriptional and Epigenetic Mechanisms Underlying Enhanced in Vitro Adipocyte Differentiation by the Brominated Flame Retardant BDE-47

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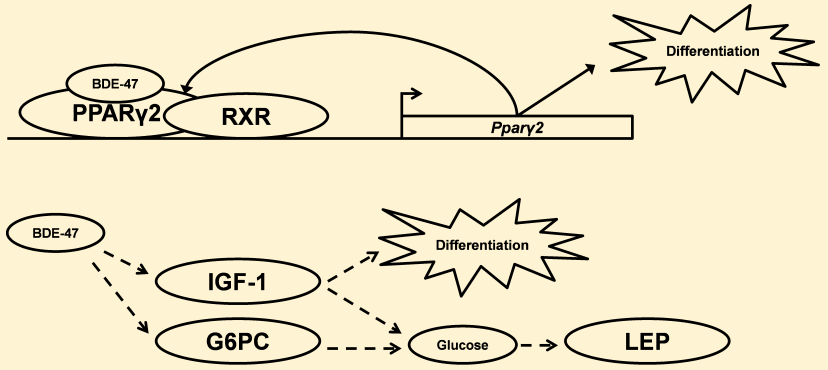
Supporting Information



ABSTRACT: Recent studies suggest that exposure to endocrine-disrupting compounds (EDCs) may play a role in the development of obesity. EDCs such as the flame retardant 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47) have been shown to enhance adipocyte differentiation in the murine 3T3-L1 model. The mechanisms by which EDCs direct preadipocytes to form adipocytes are poorly understood. Here, we examined transcriptional and epigenetic mechanisms underlying the induction of in vitro adipocyte differentiation by BDE-47. Quantitative high content microscopy revealed concentration-dependent enhanced adipocyte differentiation following exposure to BDE-47 or the antidiabetic drug troglitazone (TROG). BDE-47 modestly activated the key adipogenic transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) in COS7 cells, transiently transfected with a GAL4 reporter construct. Increased gene expression was observed for *Pparγ2*, leptin (*Lep*), and glucose-6-phophatase catalytic subunit (*G6pc*) in differentiated 3T3-L1 cells after BDE-47 exposure compared to TROG. Methylation-sensitive high resolution melting (MS-HRM) revealed significant demethylation of three CpG sites in the *Pparγ2* promoter after exposure to both BDE-47 and TROG in differentiated 3T3-L1 cells. This study shows the potential of BDE-47 to induce adipocyte differentiation through various mechanisms that include *Pparγ2* gene induction and promoter demethylation accompanied by activation of PPARγ, and possible disruption of glucose homeostasis and IGF1 signaling.

INTRODUCTION

The generally accepted cause of obesity is an imbalance between energy intake and expenditure, but this cannot account for the total increase of this disease worldwide.[1] Among other risk factors, evidence is increasing that exposure to endocrine-disrupting chemicals (EDCs), also known as obesogens, can have adverse effects on adipogenesis, lipid metabolism, and body weight as found in epidemiological, in vivo, and in vitro studies.[2] In animal studies, (perinatal) exposure to several EDCs has been related to an increase in adipose tissue and body weight, for example diethylstilbestrol (DES)[3] and tributyltin (TBT).[4] Bisphenol A (BPA) exposure has also been shown to increase body weight in rodents,[5] as well as to induce adipocyte differentiation in vitro in the 3T3-L1 murine preadipocyte differentiation model, at concentrations as low as 10 nM.[6]

We recently demonstrated a novel stimulatory adipogenic effect of BDE-47 in 3T3-L1 cells.[7] BDE-47 is a flame retardant found throughout the world in different matrixes, e.g., human

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blood, milk, dust, and various types of food.[8] BDE-47 has been shown to have endocrine-disrupting properties in vitro and in vivo.[9,10] Recent studies indicate that BDE-47 exposure also affects metabolic pathways in vivo, as an increase in body weight has been observed in male mice postnatally exposed to BDE-47, although no specific adipogenic end points were measured.[11] Additionally, prenatal exposure to BDE-47 in rats affected body weight in both male and female offspring which was accompanied by changes in several key processes in glucose homeostasis and fat metabolism exclusively for males.[5,6] Despite the recent data on increased body weight and enhanced 3T3-L1 adipogenesis by BDE-47, the molecular mechanisms behind these changes remain unknown.

Recent insights into the differentiation of 3T3-L1 cells provide a basis for understanding possible transcriptional mechanisms by which EDCs such as BDE-47 direct preadipocytes to form adipocytes. Adipocyte differentiation involves major transcriptional regulatory steps in which peroxisome proliferator-activated receptor gamma 2 (PPARγ2), an isoform of PPARγ predominantly expressed in adipose tissue, is known to be the master regulator.[14,15] Differentiation of 3T3-L1 cells is driven by two waves of transcription factors.[16] The first wave is directly activated by an adipogenic cocktail consisting of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin (MDI cocktail) and includes induction of CAAT/enhancer-binding proteins β (C/EBPβ) and C/EBPδ. Subsequently, a second wave of late-acting adipogenic transcription factors is induced, including C/EBPα and PPARγ, which in turn activate the adipogenic gene program. PPARγ binds as an obligate heterodimer with retinoid X receptor (RXR) to thousands of sites in the genome[15] and appears to be directly involved in the activation of most adipocyte specific genes, such as lipoprotein lipase (LPL), fatty acid binding protein 4 (FABP4), glucose transporter type 4 (SLC2A4), and adiponectin (ADIPOQ) as well as the PPARγ-independent adipokine leptin (LEP) (Figure 1).[14] Several studies have shown that some compounds that stimulate adipogenesis, such as tributyltin (TBT)[4] and butylparaben,[17] act via the known adipogenic pathway of PPARγ and/or RXRα activation while others, such as 2,2′4,4′,5,5′-hexachlorinated biphenyl (CB-153)[17] and bisphenol A diglycidyl ether (BADGE),[6] increase differentiation of 3T3-L1 cells through mechanisms independent of PPARγ activation.

Furthermore, EDCs may influence epigenetic processes during adipogenesis. The epigenome of the adipocyte is extensively modified as part of the transcriptional reprogramming during adipogenesis, which involves histone-modifying complexes as well as the more stable DNA methylation mark.[18−21] For instance, compared to undifferentiated 3T3-L1 cells, demethylation was observed in differentiated 3T3-L1 cells within a CpG island (CGI) at the leptin (*Lep*) promoter.[22] Hypomethylation has also been found in a regulatory region of the promoter of *Pparγ2* after differentiation of 3T3-L1 cells, which was linked to increased gene expression.[23] Increasing evidence suggests involvement of EDCs in DNA methylation mechanisms,[24] and therefore EDCs could possibly affect DNA methylation in promoter regions of genes of key adipogenic transcription factors. A direct link between exposure to adipogenic compounds and DNA methylation at promoters of specific adipogenic loci has not been reported to our knowledge, but animal studies have shown that exposure to EDCs such as DES and BPA can affect both body weight and CpG methylation at several loci.[25,26] Furthermore, we have found that differentiation of 3T3-L1 cells by EDCs such as TBT is accompanied by global demethylation.[7] In the same study, a modest decrease in global methylation was also found after exposure of 3T3-L1 cells to BDE-47, though the specific loci affected were not identified.

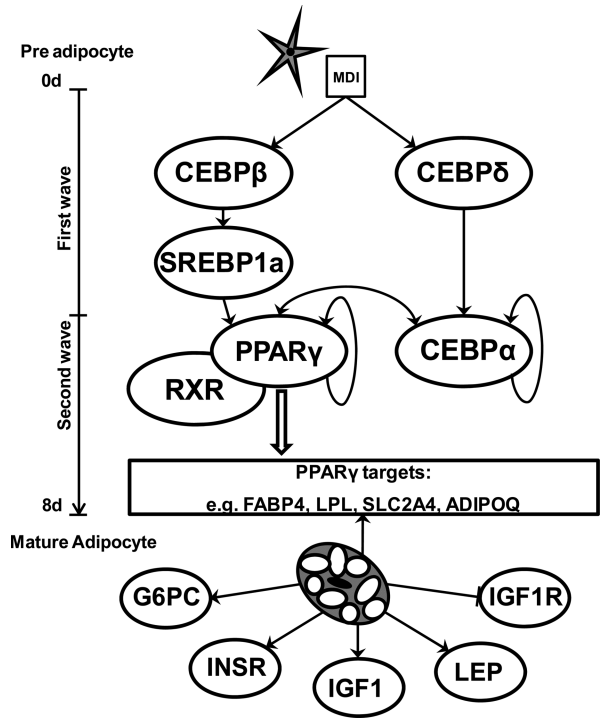

Figure 1. Genes involved in adipogenesis and analyzed in this study. During the course of differentiation, two waves of transcription factors direct preadipocytes to change into adipocytes. In 3T3-L1 cells, the first wave is induced by a cocktail of IBMX, dexamethasone, and insulin (MDI), causing enhanced transient transcription of CAAT/enhancer binding proteins, *Cebpβ* and *Cebpδ*, followed by expression of sterol-responsive element binding protein 1a (*Srebf1a*). These transcription factors induce a second wave of transcription factors (*Pparγ2* and *Cebpα*). PPARγ2 will form a heterodimer with retinoic x receptor (RXR), causing expression of PPARγ2 targets such as fatty acid binding protein 4 (*Fabp4*), lipoprotein lipase (*Lpl*), glucose transporter type 4 (*Slc2a4*), and adiponectin (*Adipoq*). Mature adipocytes will differentially express genes involved in glucose homeostasis compared to preadipocytes (e.g., increases in glucose 6 phosphatase catalytic subunit (*G6pc*), insulin growth factor 1 (*Igf1*), leptin (*Lep*), and insulin receptor (*Insr*), and a decrease in insulin growth factor receptor (*Igfr*)).[14,40−42]

Here, we examine the transcriptional and epigenetic mechanisms underlying the stimulation of 3T3-L1 adipocyte differentiation by BDE-47. Insulin-sensitizing drugs, thiazolidinediones (TZDs), were used as reference compounds in this study, as they are known to be potent ligands for PPARγ.[27] Our results indicate that the effects of BDE-47 on 3T3-L1 adipogenesis are partly mediated through activation, gene induction, and promoter demethylation of PPARγ2.

MATERIALS AND METHODS

Chemicals. The thiazolidinedione troglitazone (TROG; >98%) was obtained from Sigma Aldrich (Germany). LG268 was a gift of Dr. R. Heyman (Ligand Pharmaceuticals), and rosiglitazone (ROSI) was obtained from BioMol (Farmingdale, NY). BDE-47 was synthesized at Stockholm University and charcoal purified to remove impurities such as brominated

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dibenzofurans and dioxins and was kindly provided by Professor Åke Bergman (Stockholm University, Sweden). Compounds were dissolved in DMSO (99.9%) obtained from Acros Organics (Belgium).

3T3-L1 Cell Culture. 3T3-L1 cells (ATCC, Manassas, VA) were maintained in DMEM (high glucose, 15 mM HEPES, and glutamax) (Gibco, The Netherlands), supplemented with 1× nonessential amino acids (Gibco, The Netherlands), 10% FCS (Sigma Aldrich, Germany), and penicillin/streptomycin (Gibco, The Netherlands). Cells were subcultured twice a week at 70−80% confluence. All experiments were performed at passage 6.

Differentiation Experiments. Differentiation experiments were performed as described previously.[7] Cells were seeded in culture medium at a density of 15 000 cells/cm^2 in 6 (RNA/DNA analysis) or 48 (Cellomics analysis) well plates in a volume of 2 or 0.2 mL, respectively. For RNA/DNA analysis, 2 wells per treatment were included. For Cellomics analysis, 3 wells per treatment were used. Two days after reaching confluency (t = 0), cells were exposed to different concentrations of BDE-47, TROG, or vehicle (DMSO, 0.1%) in 2 mL (6 well) or 0.25 mL (48 well) in differentiation medium (MDI medium), consisting of 1.67 μM insulin (Roche, The Netherlands), 1 μM dexamethasone (Sigma Aldrich, Germany), and 5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Aldrich, Germany) in culture medium. Using the same conditions, the exposures were refreshed after 24 h. Two days after starting induction of differentiation, MDI medium was replaced with culture medium containing only the test compounds or vehicle. This step was repeated at day 4 and 6. Experiments were finalized at day 8. During the experiments, undifferentiated cells were used as an untreated control (undifferentiated control) and MDI-induced cells (differentiated control, 0.1% DMSO) were used as a vehicle control of basal differentiation. Two independent experiments were performed for both RNA/DNA and Cellomics analysis.

Analysis of Adipocyte Differentiation. 3T3-L1 cells were analyzed after 8 days using high-content microscopy by Cellomics Arrayscan technology (Thermo Scientific, The Netherlands). Cells were fixed in 4% paraformaldehyde (Sigma, Germany) and stained with 1 μg/mL Hoechst 33258 (Invitrogen, Grand Island, NY) and 10 μg/mL Nile Red (Sigma Aldrich, Germany) in 0.25 mL milli-Q water. Stained cells were analyzed by Cellomics Arrayscan high-content microscopy (Thermo Scientific, The Netherlands) at 10× magnification. From each well, 80 image fields were scanned and analyzed using the compartmental analysis bio application (v3.0), designed to quantify compartmentalized changes in fluorescence.[28] End points measured were total number of cells and adipocytes, adipocyte cell size, and the number of fat droplets per cell.

Gene Expression Analysis. Total RNA was isolated and purified from 3T3-L1 cells with the Nucleospin RNAII extraction kit (Macherey-Nagel, Germany). Equal amounts of RNA were converted into cDNA with the high capacity cDNA RT kit (Applied Biosystems, Grand Island, NY), according to manufacturer's recommendations. Subsequently, cDNA was diluted 20× in Milli-Q water prior to QPCR. QPCR experiments were performed in duplicate per sample on the ECO QPCR system (Illumina Inc., San Diego, CA) in 5 μL reactions, containing ABsolute QPCR SYBR Green Mix (Thermo Scientific, The Netherlands), 250 nM primer mix, and 2 μL of 20× diluted template using the following program: 95 °C for 15 min; 40 cycles of 95 °C for 15 s; 60 °C for 45 s. Primers were tested for efficiency, dimers, and nonspecific products (Supporting Information Table 1). After assessment of candidate reference genes (*Hprt*, *Nono*, and *Bactin*), *Bactin* showed the most stable Cq values relative to the amount of input RNA for cDNA synthesis and was therefore regarded as unaffected during differentiation and between the different exposures (data not shown). Expression was calculated relative to the differentiated control of day 1, as described earlier.[29]

PPARγ and RXRα Reporter Gene Assays. GAL4-hPPARγ and GAL4-hRXRα transfection assays in COS7 cells were performed as previously described.[6] ROSI and LG268 were used as a positive control for PPARγ and RXRα, respectively. BDE-47 was tested up to 33 μM in 0.1% DMSO. Each experiment contained triplicate replicates, and at least two independent experiments were performed.

Methylation-Sensitive High Resolution Melting (MS-HRM) Analysis. MS-HRM analysis was performed based on net temperature shift (NTS) calculations as described by Newman and colleagues.[30] Extended experimental procedures are found in Supporting Information. In short, DNA was bisulfite converted using the Epitect Bisulfite Kit (Qiagen, Germany) as described by the manufacturer. Different ratios of unmethylated toward fully methylated control DNA of a specific promoter region were produced to generate a calibration curve. After bisulfite conversion and PCR of control DNA and unknown samples, HRM analysis was performed on the ECO QPCR system (Illumina Inc.). Percentage methylation was calculated by interpolating unknowns in the calibration curve. Analyses were performed in duplicate on each of two independent experiments.

Direct Sequencing. To confirm HRM results, bisulfite sequencing reactions were performed with the Big Dye Terminator Kit 1.1 (Applied Biosciences), using the forward primer of leptin CGI1 (Supporting Information Table 2b) in samples from one experiment. Reaction conditions were 1 μL of 10 μM primer, 4 μL of 2.5× reaction buffer, 1 μL of premix, and 20× diluted PCR sample (from HRM reactions) up to a volume of 10 μL. Cycling conditions were 96 °C for 10 s, 59 °C for 10 s, and 60 °C for 75 s for 25 cycles. Products were purified by adding 40 μL of water and 5 μL of 3 M sodium acetate (Sigma-Aldrich, Germany) to the samples to precipitate DNA. After centrifugation (13 000 rpm, 25 min), samples were rinsed twice with ethanol (P.A., Sigma-Aldrich, Germany) and centrifuged again (13 000 rpm, 1 min). Finally, samples were dissolved in 30 μL of water and analyzed on the 3730 DNA analyzer (Applied Biosystems). Sequence Analysis software (v5.1.1, Applied Biosystems) was used to measure the peak intensity of thymidine (T) and cytosine (C) at CpG sites. The C/T ratio was calculated as a measure for methylation status of CpG sites.

Statistical Analysis. Statistical calculations were performed with GraphPad Prism software (v5.04, Graphpad Software Inc., La Jolla, CA). Data were analyzed by two-way ANOVA with treatment and experiment number as independent variables. In case no main effect or interaction effect were found for replicate experiments, congregated data of two experiments were analyzed by one-way ANOVA. Differences between treatments were analyzed by Bonferroni's multiple comparison posthoc tests ($P < 0.05$).

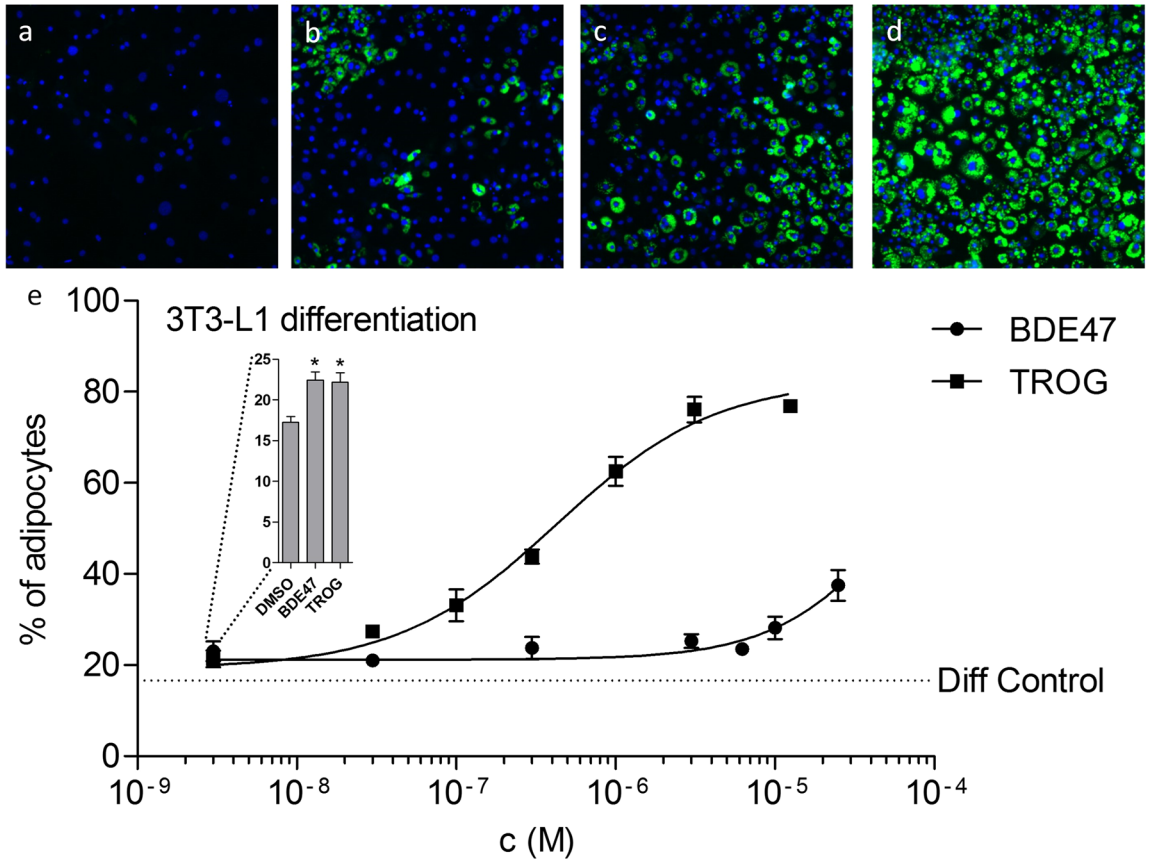

Figure 2. Quantification of 3T3-L1 differentiation with Cellomics Arrayscan technology. Nile red (green) and Hoechst (blue) staining of (a) undifferentiated cells, and differentiated cells exposed to (b) differentiated control (DMSO), (c) BDE-47 (25 μM), and (d) TROG (1 μM). (e) Concentration-dependent effect of BDE-47 and TROG on adipocyte differentiation, with a cutout at 3 nM showing significant enhancement of differentiation. (Graph is presented as mean ± SD from one out of two reproducible experiments; cutout is presented as mean ± SD of congregate data of two experiments).

RESULTS

Adipocyte Differentiation. We developed a high throughput analysis system for measuring adipocyte differentiation in 48-well plates, using a double Nile Red/Hoechst stain to quantify the increase in adipocyte cell number by TROG and BDE-47 exposure compared to the undifferentiated and differentiated controls (Figure 2, parts c,d vs a,b). In two independent experiments, a reproducible concentration–response-related increase was found by BDE-47 and TROG both in cell proliferation which occurs during the first wave of transcription factor induction (data not shown) and differentiation to adipocytes after 8 days (Figure 2e). We optimized the 3T3-L1 differentiation conditions to obtain a relatively low level of differentiation in the differentiated controls (17%), which allowed for enhanced discrimination of the effects of the test chemicals on adipocyte differentiation (up to 38% and 76% for BDE-47 and TROG, respectively) (Figure 2e). Based on congregated data of two experiments, a significant increase in adipocytes was found compared to the differentiated control at the lowest level tested, namely 3 nM (17.2% for differentiated control vs 22.4% and 22.1% for BDE-47 and TROG, respectively) (Figure 2e). No significant effect was found on adipocyte cell size by either TROG or BDE-47 (data not shown).

Transactivation of PPARγ and RXRα. The observed increase in adipocyte differentiation by BDE-47 in 3T3-L1 cells led us to examine the possibility that BDE-47 may be a ligand for PPARγ and/or RXRα. In transient transfection assays in COS7 cells with GAL4-hPPARγ, concentration-dependent PPARγ activation was observed for BDE-47 up to 33 μM, though the maximum induction (up to 3×) was much lower compared to the 150× induction by the TZD reference compound rosiglitazone (ROSI) (Figure 3a). Exposure to higher concentrations up to 100 μM BDE-47 confirmed these results by showing a stronger activation of PPARγ (7×, data not shown). In GAL4-RXRα-transfected COS7 cells no activation of RXRα was observed after BDE-47 exposures, though the reference RXRα ligand LG268 showed distinct concentration-related effects (Figure 3b).

Expression of Adipogenic Genes during Differentiation. Because we observed only weak activation of PPARγ by BDE-47, the transcriptional mechanism of action underlying BDE-47-enhanced adipocyte differentiation was further studied. Expression of several key genes involved in 3T3-L1 adipogenesis was measured at various time points during induced differentiation by BDE-47 or TROG exposures (Figure 4). Expression was calculated relative to the differentiated control of day 1. To obtain maximum differences in gene expression, BDE-47 and TROG were tested at concentrations exhibiting the highest compound specific adipocyte differentiation with no visual cytotoxic effects, i.e., 25 μM and 1 μM, respectively. Furthermore, these concentrations showed no cytotoxic effects in lactate dehydrogenase leakage (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as-

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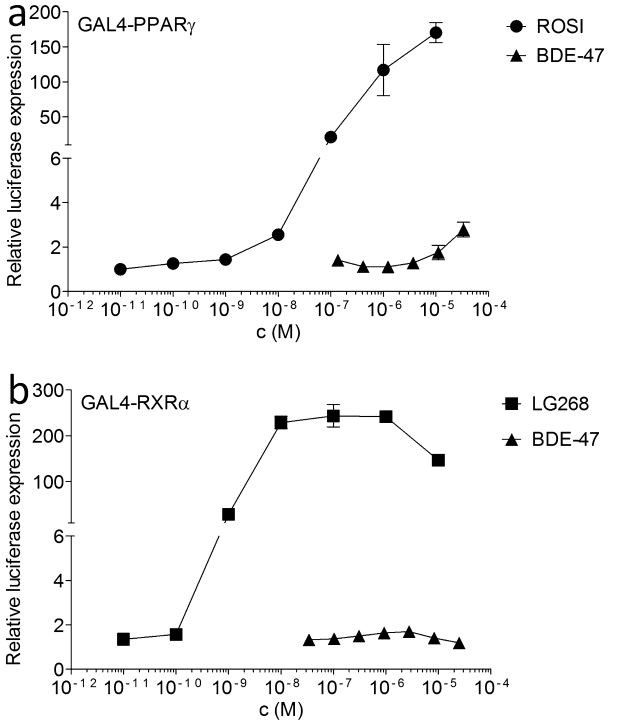

Figure 3. Transactivation of PPARγ and RXRα. (a) Rosiglitazone (ROSI) shows over 150× higher induction in luciferase expression relative to the vehicle control (DMSO). BDE-47 shows weak but significant activation of GAL4-PPARγ up to 2.5×. (b) Although the known RXRα activator LG268 induces luciferase expression over 200 times, no activation is observed with BDE-47. Data are reported as relative luciferase expression over vehicle (0.1% DMSO) controls (mean ± SD) for triplicate samples (three biological replicates), and similar results were obtained in additional experiments.

says following exposure to BDE-47 for 72 h in undifferentiated 3T3-L1 cells.[7] When first considering the differentiated control (Figure 4), mRNA levels of *Cebpβ* and *Cebpδ* both increased after day 1, followed by an increased expression of *Srebf1a*. mRNA levels of *Pparγ2* and *Cebpα* were elevated after the initiation phase and were highly expressed during the whole process of differentiation. The expression of PPARγ targets *Lpl*, *Slc2a4*, *Fabp4*, and *Adipoq* was enhanced at day 3 and 8. During the course of differentiation, increases in mRNA were observed for *G6pc*, *Lep*, *Igf1*, and *Insr*, whereas a decrease was observed in *Igf1r*.

Exposure of 3T3-L1 cells to BDE-47 or TROG during differentiation only modestly affected mRNA expression of the transcription factors during the first 2 days of differentiation (Figure 4). A slight increase was observed for both *Cebpβ* and *Rxrα* expression in response to both BDE-47 and TROG compared to differentiated control at day 2. Expression of *Cebpβ* was significantly higher for BDE-47 than the differentiated control at this time point, while the expression of *Rxrα* was significantly higher for TROG. More pronounced differences in gene expression between the two chemicals were found at later stages in differentiation. Although TROG is a more potent inducer of adipocyte differentiation than BDE-47, only BDE-47 induced an increase in *Pparγ2* mRNA expression after 8 days. However, this difference in *Pparγ2* expression was not reflected in the PPARγ targets (*Cebpα*, *Lpl*, *Slc2a4*, and *Fabp4*). Of those targets, TROG exhibited significant increases in expression at an earlier stage (at 2 and 3 days) and at a level higher than that of BDE-47. *Adipoq* mRNA levels did not differ between exposures. Expression analysis of both *G6pc* and *Lep* showed significant enhancement exclusively by BDE-47 compared to the differentiated control (Figure 4).

DNA Methylation Analysis of the *Pparγ2* and *Lep* Promoter. To determine if the increased *Pparγ2* expression at 8 days by BDE-47 exposure was linked to the methylation status of the *Pparγ2* promoter region, we employed methylation-sensitive high resolution melting (MS-HRM).[30] A region in the *Pparγ2* promoter with three CpGs sites between −337 and −192 base pairs from the transcription start site (TSS) was selected based on a previous study (Figure 5a).[23] This region, located within a DNase I hypersensitive site,[18] represents an open chromatin structure, where proteins such as transcription factors are able to bind and potentially regulate *Pparγ2* expression. A calibration curve consisting of control DNA mixtures with different ratios of unmethylated vs fully methylated CpGs was used to quantify the methylation status of the region, based on net temperature shift (NTS) calculations (Figure 5b). MS-HRM analysis revealed significant demethylation of the three CpGs present in this region after differentiation with BDE-47 and TROG compared to undifferentiated cells (Figure 5c).

Because *Lep* mRNA expression was elevated by BDE-47 exposures compared to TROG and differentiated control, MS-HRM analysis was also performed on the *Lep* promoter. The promoter region of *Lep* contains three CpG islands (CGIs), as defined by the NCBI genome viewer (http://www.ncbi.nlm.nih.gov/). Two of them (CGI1 and 2) were analyzed by MS-HRM (Figure 6a). A high methylated state (approximately 70%) and no significant differences between the exposures were observed in the analyzed region of CGI1 (Figure 6c). These results were validated by direct sequencing of the PCR products obtained from the MS-HRM analysis, yielding a similar trend for the methylated controls (0%, 50%, and 100% methylated) at the eight analyzed CpG sites and confirming that no differences in methylation were observed between the different exposures (Figure 6d). The second CGI, located at a more distal region upstream from the *Lep* promoter, also showed no differences between the exposures (Figure 6f).

DISCUSSION

Enhanced Adipocyte Differentiation. Increasing evidence suggests that exposure to EDCs may play a role in the worldwide epidemic of obesity through various mechanisms including effects on adipocyte differentiation.[2] This study reveals that BDE-47, an endocrine-disrupting brominated flame retardant found ubiquitously in the environment, induces adipocyte differentiation in 3T3-L1 cells at concentrations as low as 3 nM. We developed a novel quantification technique using Cellomics Arrayscan technology, which represents a significant step forward from widely used techniques such as Oil Red O, or Nile Red lipid staining combined with spectrophotometric analysis or fluorescence-activated cell sorting (FACS). The Cellomics Arrayscan allowed fast and accurate measurements of cell numbers, cell size, and cell type (adipocytes) that parallels the accuracy of FACS and the high throughput capacity of staining techniques, which allows us to screen many compounds and environmental extracts for adipogenic properties.

BDE-47 levels in North America are among the highest reported throughout the world, generally 1 magnitude higher than elsewhere and with no indication of decline.[31] Levels

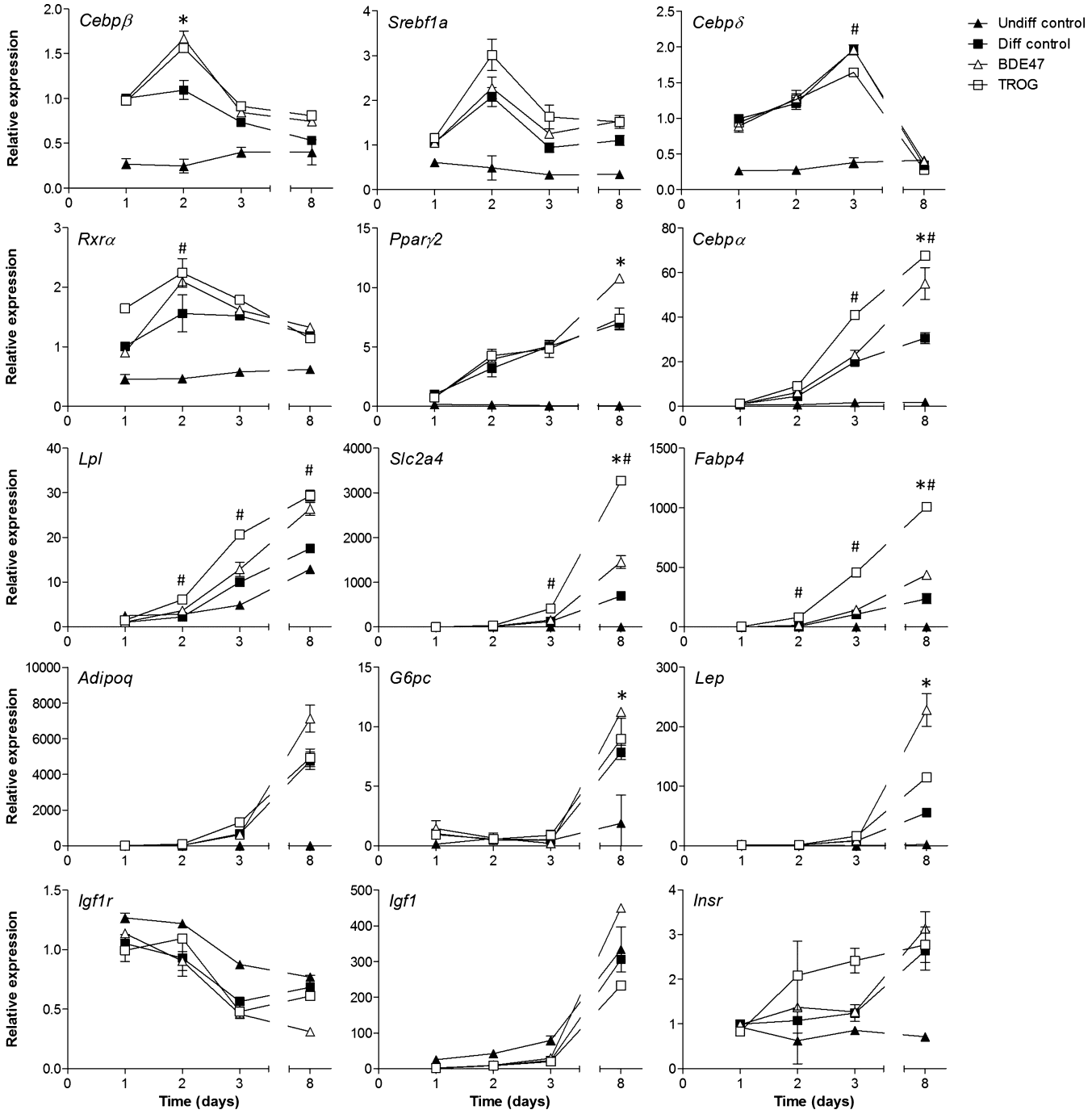

Figure 4. Gene expression analysis of selected targets involved in 3T3-L1 adipocyte differentiation in undifferentiated (Undiff control) or differentiated cells following exposure to vehicle (0.1% DMSO, Diff control), BDE-47 (25 μM), and TROG (1 μM). Initiation of differentiation begins with a transient increase in expression of *Srebf1a*, *Cebpβ*, *Cebpδ*, and *Rxrα*, followed by an increase of *Pparγ2* and *Cebpα*. Consequently, adipocyte specific markers *Lpl*, *Slc2a4*, *Fabp4*, and *Adipoq* are increased. Genes involved in glucose homeostasis are altered in mature adipocytes, with increases observed for *G6pc*, *Lep*, *Igf1*, and *Insr*, and a decrease in expression of *Igf1r*. (Figures represent one out of two reproducible experiments, calculated relative to the differentiated control of day 1 (mean ± SD *BDE-47 significantly different compared to differentiated control, #TROG significantly different compared to differentiated control. Significance was calculated averaging two experiments ($P < 0.05$)).

reported in blood from US citizens range from 0.63 to 46 ng/g (1.1 to 81.5 pmol/g or 0.008 to 0.6 nM) lipid weight, and these levels indicate a minimal margin of exposure of only 5 when compared to the 3 nM concentration used in the 3T3-L1 medium, based on an average lipid content in blood of 0.7%.[8] Furthermore, the highest reported level of BDE-47 found in serum from children working at waste disposal sites in Managua (Nicaragua) was 384 ng/g (680 pmol/g) lipid weight (0.4% lipid), corresponding to 2.8 nM in serum.[32] Because these levels are close to the concentrations used in this study, the results cannot be neglected.

PPARγ Activation. Although many EDCs are able to induce adipocyte differentiation in 3T3-L1 cells, the underlying mechanisms have been elucidated for only a few. Here, we show that BDE-47 weakly activates PPARγ, but not RXRα, in transient transfection assays. The activation of PPARγ is likely

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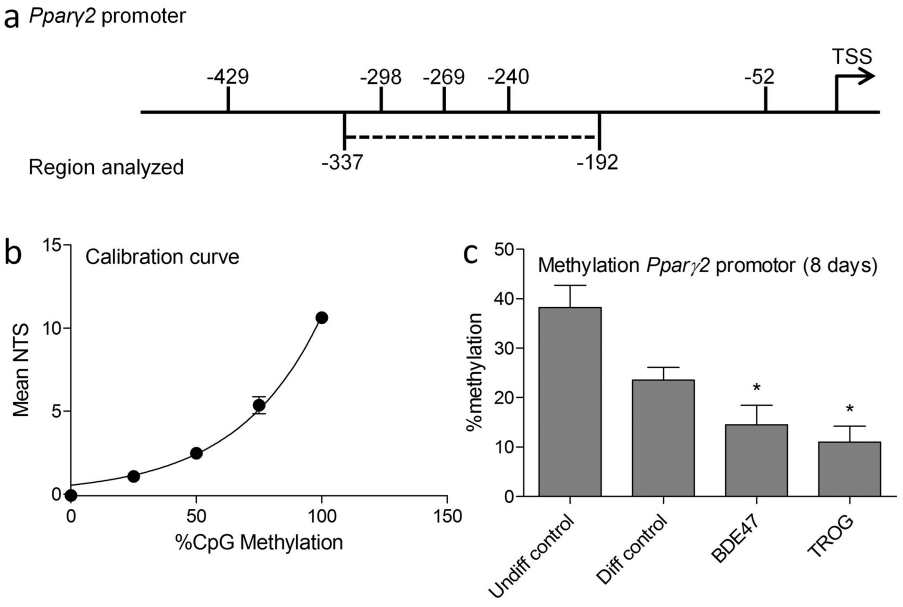

Figure 5. Methylation analysis of the *Pparγ2* promoter. (a) A schematic overview of the analyzed region showing three CpG sites at positions −240, −269, and −298 from the transcription start site (TSS). (b) A typical calibration curve of percentage methylation (%CpG) plotted against the mean net temperature shift (NTS) was used to interpolate samples (c) Methylation of the *Pparγ2* promoter in DNA samples from undifferentiated 3T3-L1 and differentiated cells exposed to vehicle control (0.1% DMSO), BDE-47 (25 μM), and TROG (1 μM). (Data for c are shown as the mean of two independent experiments ± SD *significantly different from undifferentiated cells, $P < 0.05$).

to be causally related to the significantly increased expression of many PPARγ target genes after exposure to 25 μM BDE-47 during differentiation. In previous studies, alkylated metals such as tributyltin (TBT) have been shown to activate the PPARγ/RXRα heterodimeric complex in murine and human adipose-derived stromal stem cells[4,33] and in 3T3-L1 cells.[4] However, other compounds such as BADGE and BPA,[6] and PCB-153,[17] have been shown to induce adipogenesis in 3T3-L1 cells by a PPARγ/RXRα independent mechanism. These results indicate that EDCs may exert adipogenic effects via pathways other than activation of key adipogenic transcription factors.

Differential Expression and Promoter Demethylation of PPARγ. Because only weak activation of PPARγ by BDE-47 was observed during transient transfection experiments, differential expression of several adipogenic target genes was examined to investigate other transcriptional routes by which BDE-47 could exhibit its adipogenic action. Generally, genes involved in the initiation of adipocyte differentiation and maturation exhibited the same trend for both BDE-47 and the prototypical PPARγ ligand TROG compared to the differentiated control. However, the increased expression of *Pparγ2* at 8 days of differentiation, exclusively after BDE-47 exposure, was an unexpected finding. Though further analysis is needed, the difference of *Pparγ2* expression between BDE-47 and TROG may be explained by the negative feedback of TROG on *Pparγ2* expression which has been reported in mature 3T3-L1 adipocytes.[34] A similar repression may not occur in response to BDE-47.

We investigated if the increased gene expression of *Pparγ2* found after BDE-47 exposure was related to a decreased methylation status of the *Pparγ2* promoter. It has been established that the chromatin structure of the *Pparγ2* promoter is more open in differentiated than undifferentiated adipocytes and that several CpG sites are demethylated in the promoter region of *Pparγ2*, which is linked to increased gene expression.[23] We quantified the methylation status of three CpG sites on the *Pparγ2* promoter and confirmed that the promoter is hypomethylated after differentiation which is concurrent with the increased gene expression of *Pparγ2*. Importantly, we show for the first time that both BDE-47 and TROG exposure resulted in significant lower methylation levels of the *Pparγ2* promoter after differentiation. As the methylation status of the *Pparγ2* promoter was similar between BDE-47 and TROG, despite the differences in gene expression, we hypothesize that additional loci amenable to methylation or other epigenetic processes involved in *Pparγ2* expression could be affected by BDE-47, such as histone modifications.[21,23]

Disturbed Glucose Homeostasis. Higher levels of *G6pc* mRNA were found in differentiated 3T3-L1 cells after BDE-47 exposures compared to TROG. *G6pc* encodes for the catalytic subunit of glucose-6-phosphatase and is responsible for maintaining intercellular glucose levels.[35] Additionally, we found that *Igf1* mRNA levels increased up to 400 fold during differentiation and were highest in BDE-47 exposed cells. It has been shown that either insulin or IGF1 is essential to achieve proper differentiation in 3T3-L1 cells in a dose-dependent matter.[36] Increased levels of IGF1 and increased recovery rates of blood glucose in plasma have been found in male rats perinatally exposed to BDE-47, accompanied by increased body weight.[12] The increased expression of *G6pc* found in livers of these animals was suggested to be linked to the increased glucose uptake.[13] The elevated *G6pc* mRNA levels found with BDE-47 exposure in this study, together with the increased *Igf1* mRNA levels during differentiation, suggests a similar mechanism of glucose homeostasis disruption in 3T3-L1 cells. The elevated expression of *G6pc* may also play a role in the increased *Lep* expression observed after BDE-47 exposure. Higher levels of *G6pc* cause higher levels of cytoplasmic glucose,[35] which subsequently could lead to increased LEP levels. Accordingly, enhanced *Lep* expression and leptin release in 3T3-L1 adipocytes has been observed after glucose exposure.[37] Further research is needed to confirm a possible

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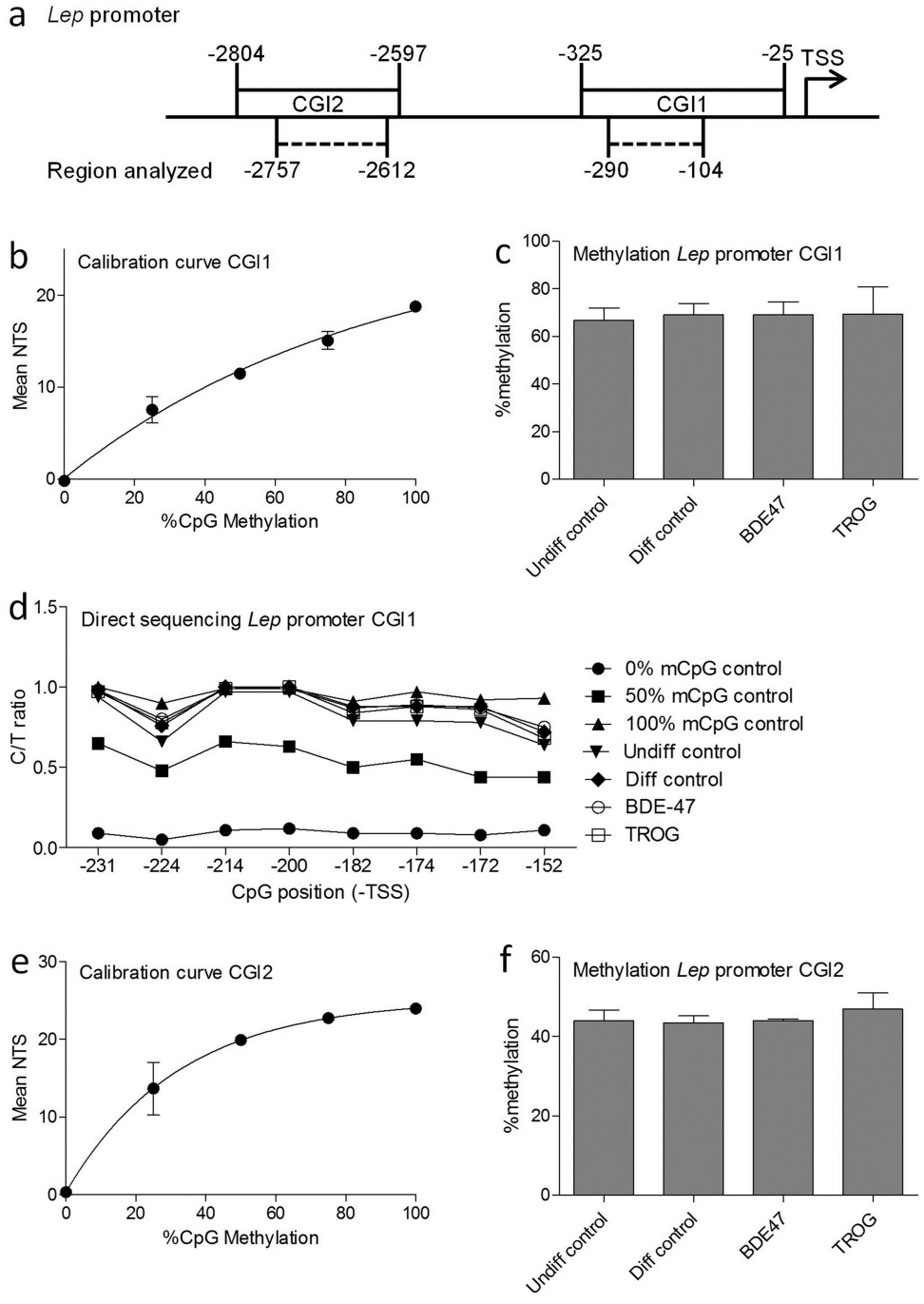

Figure 6. MS-HRM analysis of two CpG islands (CGIs) of the *Lep* promoter after exposure to BDE-47 (25 μm) and TROG (1 μM). (a) A region between −104 and −290 base pairs from the TSS was analyzed in CGI1, which included 13 CpG sites. Another, more distal, CGI (CGI2) was analyzed in a region ranging from −2612 to −2757 base pairs (6 CpG sites) from the TSS. (b,c) Calibration curve and the methylation status of CGI1. (d) Direct sequencing results of 8 CpG sites in the first CGI of the *Lep* promoter. (e, f) Calibration curve and methylation status of CGI2. Data for c and e are shown as the mean of two independent experiments ± SD. Part d was performed on duplicate samples of one experiment.

link between BDE-47-mediated elevation of intracellular glucose levels, *G6pc* expression, and leptin levels.

Changes in CGI methylation on the promoter of *Lep*, induced either directly by BDE-47 or indirectly via enhanced glucose levels, could have been a possible mechanism leading to increased *Lep* gene expression, because hypomethylation of CGIs at promoter regions of genes is associated with gene expression, whereas hypermethylation is associated with long-term silencing of genes.[38] Demethylation of CGI1 of the *Lep* promoter after 3T3-L1 differentiation has been previously observed.[22] However, in this study, *Lep* promoter methylation was unchanged and highly methylated in all treatments and in both undifferentiated and differentiated 3T3-L1 cells, despite the dramatic increase in gene expression of *Lep* after differentiation. In concordance with our results, a recent animal study also failed to show a relationship between *Lep* expression and DNA methylation in adipose tissue.[39] We infer that other mechanisms mediated by BDE-47, such as upstream transcription factor regulation or other epigenetic mechanisms (e.g

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histone modifications), could be responsible for increased *Lep* expression.

Our study shows adipogenic effects of the ubiquitous environmental contaminant BDE-47 at low nanomolar concentrations and identifies putative mechanisms underlying the enhanced 3T3-L1 cell differentiation by BDE-47. We propose that BDE-47 induces adipocyte differentiation in vitro by increasing expression of *Pparγ2* while weakly activating PPARγ. BDE-47 also leads to decreased methylation of the *Pparγ2* promoter, which may play a role in the increased *Pparγ2* expression. A possible second mechanism suggests a role for BDE-47 in disruption of glucose homeostasis and IGF1 signaling by increasing levels of IGF1 and G6PC, which may ultimately result in enhanced *Lep* expression. Given the urgent need to understand how exposure to chemicals may influence obesity, the results found in this study provide new insights into the complex transcriptional mechanisms that are associated with enhanced adipogenesis by EDCs. This study clearly shows that EDC exposure may influence epigenetic events in vitro, which warrants further study in vivo. Our findings indicate the importance of further investigation of the obesogenic effects of brominated flame retardants, given the continued exposure of humans to this class of chemicals.

■ ASSOCIATED CONTENT

Supporting Information

A detailed description of the methylation analyses used in this study (MS-HRM and direct sequencing). Supplemental Figure 1 shows significant enhancement of adipocyte differentiation following exposures to BDE-47 and TROG. Supplemental Table 1 contains all primer sequences used in QPCR experiments. Supplemental Table 2 contains all primer sequences for methylation analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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