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Research Article

Tetrabromobisphenol-A Promotes Early Adipogenesis and Lipogenesis in 3T3-L1 Cells

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ABSTRACT

Tetrabromobisphenol A (TBBPA) is the most common flame retardant used in electrical housings, circuit boards, and automobiles. High-throughput screening and binding assays have identified TBBPA as an agonist for human peroxisome proliferator-activated receptor gamma (PPARγ), the master regulator of adipogenesis. TBBPA has been suggested to be an obesogen based on *in vitro* cellular assays and zebrafish data. We hypothesized that exposing preadipocytes to TBBPA could influence adipogenesis via genes other than those in the PPARγ pathway due to its structural similarity to bisphenol A, which demonstrates varied endocrine disrupting activities. Mouse-derived 3T3-L1 preadipocytes were induced to differentiate and continually treated with TBBPA for 8 days. High-content imaging of adipocytes displayed increased adipocyte number and lipid accumulation when treated with TBBPA. TBBPA exhibited weak induction of mPPARγ, with an AC_{50} of 397 μM. Quantitative PCR revealed that TBBPA exposure increased early expression of genes involved in glucocorticoid receptor (GR) signaling and PPARγ transcriptional activation, as well as upregulating downstream genes needed for adipocyte maintenance and nontraditional ER signaling, such as *Gpr30*. Additionally, *Pref1 and Thy1*, inhibitors of differentiation, were downregulated by some concentrations of TBBPA. Furthermore, proliferating preadipocytes treated with TBBPA, only prior to differentiation, exhibited increased adipocyte number and lipid accumulation after 8 days in normal culture conditions. In conclusion, TBBPA influenced gene expression changes in GR, nontraditional ER, and known adipogenic regulatory genes, prior to PPARγ expression; effects suggesting early programming of adipogenic pathways.

Key words: tetrabromobisphenol A; TBBPA; endocrine disruptor; obesity; obesogen; PPARγ; 3T3-L1; adipocyte; lipid accumulation; adipogenesis.

The prevalence of overweight (70.4%) and obesity (37.7%) in adult U.S. individuals (age ≥ 20 years) has reached an alarming rate (Flegal *et al.*, 2016; Ogden *et al.*, 2015). More concerning is the prevalence of obesity among U.S. children; affecting 17% of 2–19-year-olds (Ogden *et al.*, 2015). Overweight and obese individuals are at risk for a number of chronic diseases, including: heart disease, type 2 diabetes, high blood pressure, and some cancers (Mokdad *et al.*, 2003). High caloric consumption, lack of physical activity, and genetic predisposition are classic factors influencing obesity. Obesity and its associated adverse health effects have had a costly impact on healthcare burden in the European Union (Legler *et al.*, 2015), and there is mounting evidence that environmental factors encountered during early life may contribute to these negative influences on lifelong health. Genetics cannot diverge fast enough to account for the increased prevalence of obesity over the last decades; therefore, environmental factors are suggested to be responsible for at least part of the increased incidence (Heindel, 2003; Thayer *et al.*, 2012). Environmental chemicals that have been associated with increased weight gain or obesity in epidemiological or

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experimental studies include persistent organic pollutants, solvents, organophosphates, phthalates, bisphenol A (BPA; and its analogues), and estrogenic compounds (Cano-Sancho *et al.*, 2017; Gore *et al.*, 2015; Heindel *et al.*, 2017; Nappi *et al.*, 2016).

Numerous environmental chemicals mimic natural hormones and ligands that facilitate adipocyte differentiation by affecting nuclear receptors (NRs) and other transcription factors (Grun and Blumberg, 2006; Heindel *et al.*, 2017; Matsushima *et al.*, 2007; Molina-Molina *et al.*, 2013; Riu *et al.*, 2011b). Identification of such chemicals and their modes of action is an emerging area of research. As part of a collaborative project in the National Toxicology Program, Tox21 phase II high throughput screening data was used to identify environmental chemicals of potential interest linked to obesity-related outcomes. Tetrabromobisphenol A (TBBPA) was identified as a chemical that exhibited agonist activity in high throughput screening assays for some of the theorized mediators of adipogenesis or lipid accumulation; peroxisome proliferator-activated receptor gamma (PPARγ), retinoid X receptor alpha (RXRα), and glucocorticoid receptor (GR) (Tox21 Concentration Response Browser, NTP https://sandbox.ntp.niehs.nih.gov/tox21-curve-visualization/).

TBBPA is the most commonly used brominated flame retardant and is found in epoxy resin-containing plastic products (ie, electronics), building materials, paints, textiles, and household dust (Dagani *et al.*, 2000; Riu *et al.*, 2011a; Shaw *et al.*, 2010). TBBPA is readily absorbed through the gastrointestinal tract and is primarily excreted in the feces (Hakk and Letcher, 2003). Like BPA, TBBPA is considered an environmental contaminant and has been detected globally in human blood [0.24–19.8 ng/g and 0.003–0.09 ng/ml; (Cariou *et al.*, 2008; Covaci *et al.*, 2009; Dirtu *et al.*, 2008)], breast milk [0.06–37.34 ng/g; (Carignan *et al.*, 2012; Cariou *et al.*, 2008; Shi *et al.*, 2013)], and adipose tissue (0.048 ng/g) (Covaci *et al.*, 2009). Reports from China suggest that TBBPA exposure is rising, as mean breast milk concentrations rose from 0.933 ng/g (Shi *et al.*, 2009) to means near 5 ng/g (Shi *et al.*, 2013). Other reports suggest that typical means of estimating child exposure are flawed, as TBBPA levels were considerably higher in breast milk than estimates of burden based on maternal blood measurements (Abdallah and Harrad, 2011; Cariou *et al.*, 2008). The estimated half-life of TBBPA in rodents and humans was fairly similar; less than 3 days (ranging from 10 h in the liver to 70 h in adipose tissue) in rodents, and 2.2 days in human serum (Geyer *et al.* 2004; Hagmar *et al.*, 2000; Hakk and Letcher, 2003).

Several studies have confirmed that TBBPA can disrupt thyroid signaling and has diminutive estrogenic potency (Kitamura *et al.*, 2002; Meerts *et al.*, 2000) giving TBBPA its endocrine disrupting compound (EDC) status. Recent studies in human, zebrafish, and Xenopus, have shown that TBBPA is a PPARγ agonist and can interact with its ligand-binding domain (Riu *et al.*, 2011a). TBBPA can increase gene expression of both the CCAAT/enhancer binding protein-alpha (*Cebpa*) and *Pparg*, and late (ie, fatty acid-binding protein-4, *Fabp4*) markers of adipogenesis in 3T3-L1 cells at terminal differentiation (Akiyama *et al.*, 2015; Riu *et al.*, 2011a). In addition, perinatal exposure to BPA alters adipogenesis in rats, presumably through PPARγ activation (Somm *et al.*, 2009). Growing evidence illustrates that several EDCs that promote adipogenesis may be acting via PPARγ (Heindel *et al.*, 2017). To further investigate mode of action and early effects of TBBPA during adipogenesis, we utilized the 3T3-L1 murine preadipocyte cell line; a very well characterized model for the study of the adipocyte differentiation process (Green and Kehinde, 1975). *In vitro*, exposure of confluent 3T3-L1 preadipocytes to a differentiation-inducing cocktail (MDI), a combination of methylisobutylxanthine (a cAMP phosphodiesterase inhibitor), dexamethasone (glucocorticoid), and insulin, triggers a cascade of transcriptional events (Rosen *et al.*, 1979). During the early stages of differentiation, MDI triggers a rapid, yet transient, induction of two CCAAT/enhancer-binding protein transcription factors, C/EBPβ and C/EBPδ, which induce expression of required genes for lipid production and differentiation, such as C/EBPα and PPARγ (Farmer, 2005; Rosen and MacDougald, 2006).

Prior studies of the adipogenic/lipogenic effects of TBBPA in human or mouse cell models had multiple limitations; they simply focused on TBBPA induction of PPARγ, have failed to evaluate the ability of TBBPA to initiate differentiation, and/or have used semi-quantitative methods to determine effects on adipogenesis or lipid accumulation (Kassotis *et al.*, 2017; Riu *et al.*, 2011a,b; Woeller *et al.*, 2015). This study aimed to define the early signaling events, prior to PPARγ induction, which are triggered by TBBPA in differentiation of 3T3-L1 preadipocyte cells to mature, lipid-producing adipocytes. To quantitatively evaluate TBBPA-related effects on adipogenesis we have used high-content image analysis, quantitative PCR and receptor activation assays.

MATERIALS AND METHODS

3T3-L1 cell culture

3T3-L1 cells were a kind gift provided by Dr Phillip H. Pekala (East Carolina University, North Carolina), who received them originally from Dr Howard Green, in whose lab the cell line originated (Green and Meuth, 1974; Green and Kehinde, 1975). 3T3-L1 cells were supplied at passage +2, then populated/maintained in preadipocyte media containing: high glucose Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/l glucose) (Life Technologies, Gibco, Carlsbad, California) and supplemented with 10% newborn bovine calf serum (Gemini, Sacramento, California), 10 000 U/ml penicillin/streptomycin (Life Technologies), 4.4 mg/l calcium pantothenate (Sigma-Aldrich, St. Louis, Missouri), and 8.8 mg/l biotin (Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere with 5% v/v CO_2. 3T3-L1 cells were subcultured at 70–80% confluence and grown on BD Falcon-Corning #353003 (Corning Inc., Tewksbury, Massachusetts) tissue culture dishes.

Differentiation experiments

Differentiation experiments were performed as follows: low passage number (passages +5 through +10) cells were seeded onto BD Falcon-Corning 96-well plates with black sides and clear flat bottom (Corning Inc.) at a density of 1.4×10^4 cells per well in a volume of 0.2 ml preadipocyte media. Two days after reaching visual confluence (d = 0), preadipocytes were exposed to TBBPA (log dilutions from 1×10^{-5}M to 1×10^{-12}M), vehicle (0.05% DMSO), or rosiglitazone (ROSI; 1×10^{-6} M) (Cayman Chemical, Ann Arbor, Michigan) in quadruplicates in a volume of 0.2 ml *adipocyte maintenance media* containing: high glucose DMEM supplemented with 10% fetal bovine serum (Gemini), 10 000 U/ml penicillin/streptomycin (Gibco), 4.4 mg/l calcium pentothenate, and 8.8 mg/l biotin. Concurrently, the other 4 wells per column were induced to differentiate into adipocytes by treating them with a differentiation cocktail (MDI) consisting of: 125 μM methylisobutylxanthine, 0.25 μM dexamethasone, and 2.5 mg/l insulin (Sigma-Aldrich). Our MDI contained 25% the concentration of MDI as previous reports (Cao *et al.*, 1991;

Pekala *et al.*, 1983; Riu *et al.*, 2011a) to create a submaximal adipogenic response, allowing us to quantitate increases above MDI (and potentially decreased response) due to treatment. Treated media was removed and TBBPA serial dilutions plus media were repeated on d = 2, d = 4, and d = 6 to mimic a chronic exposure; wells that received MDI treatment on d = 0 were additionally supplemented with 2.5 mg/l insulin on d = 2 and 0.625 mg/l insulin on d = 4. On d = 6 cells received adipocyte maintenance media (and respective treatments) only.

High-content imaging analysis of adipocyte differentiation

3T3-L1 cells were analyzed on d = 8 of the experiment using high-content microscopy with an inverted automated epifluorescent microscope (ImageXpress® Micro by Molecular Devices, Sunnyvale, California). Media was removed and cells were rinsed in 1 × PBS (Gibco), and were fixed in 4% paraformaldehyde (Sigma-Aldrich). Fixative was removed and cells were rinsed and stored in 1 × PBS overnight at 4°C. Cells were then co-stained with a solution of 0.3 μM Hoechst 33342 (nuclear fluorescent stain) (ThermoFisher Scientific, Waltham, Massachusetts) and 0.3 μM Nile Red (lipid droplet fluorescent stain) (Sigma-Aldrich) in 1× PBS. Stained cells were washed twice with 1× PBS and imaged at 20× magnification using the ImageXpress® Micro in 0.2 ml of 1 × PBS. Lipid droplets were identified using a FITC filter channel and nuclei were identified using a DAPI filter channel. Exposure times were automatically adjusted to each plate using MetaXpress® (Molecular Devices) software and were consistent over the entire plate (controls and treatment groups). Images were analyzed using the MetaXpress® image based multi-wavelength cell scoring application module. The mean lipid droplet area (μm^2) and the mean nuclear counts were determined from 9 images per well, $n = 4$ replicate wells (up to 36 images used per group). Endpoints measured were total mean number of cells, mean number of adipocytes, and mean lipid droplet area (μm^2) per adipocyte nuclei to assess lipid accumulation. Three separate controls are incorporated in the data analysis: (1) vehicle control group (0.05% DMSO) consists of undifferentiated preadipocyte cells (uninduced), (2) induced MDI group (MDI + 0.05% DMSO) were used as a positive control for basal differentiation (induced), and (3) ROSI treated cells were used as a positive control for a PPARγ agonist-induced differentiation (ROSI ± MDI, 0.05% DMSO). ROSI response above controls was required for a plate to be included in the study. Data are presented as a scatterplot of the 4 replicates. *Black lines*: mean ± SEM of individual groups obtained from GraphPad Prism 7.01 (GraphPad Software Inc., San Diego, California). *Dotted line*: mean vehicle control (uninduced). *Dash line*: mean induced MDI control.

Transient transfection and receptor activation analysis

pCMX-Gal4, pCMX-Gal4–mPPARγ, and pCMX-Gal4-hRXRα constructs were previously described (Grun *et al.*, 2006). pCMX-hGR and MMTV-Luc constructs were also previously described (Hollenberg and Evans, 1988). Transient transfections were performed in COS7 cells as described (Chamorro-Garcia *et al.*, 2012; Janesick *et al.*, 2016). Briefly, COS7 cells were seeded at 15 000 cells per well in 96-well tissue culture plates in 10% newborn bovine calf serum in DMEM. The following day, cells were transfected in Opti-MEM® at ∼ 90% confluency. One microgram of CMX-GAL4 effector plasmid was co-transfected with 5 μg TK-$(MH100)_4$–luciferase reporter and 5 μg of CMX-β-galactosidase transfection control plasmids in Opti-MEM using Lipofectamine® 2000 reagent (Invitrogen™ Life Technologies, Carlsbad, California), following the manufacturer's recommended protocol. After overnight incubation, the medium was replaced with DMEM plus 10% resin charcoal stripped FBS plus TBBPA (33.33, 11.1, 3.7 1.23, 0.41, and 0.14 μM) or ROSI (10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM) for an additional 24 h. DMSO concentration was maintained at 0.05% across all chemical treatments. Cells were lysed and assayed for luciferase and β-galactosidase activity as described previously (Forman *et al.*, 1995). All transfections were performed in triplicate in three separate experiments. Data are reported as fold induction over vehicle (0.05% DMSO) controls ± standard deviation using standard propagation of error. AC_{50} (half-maximal activation concentration) were obtained using nonlinear regression variable slope curve fit analysis using GraphPad Prism 7.01 (La Jolla, California). TBBPA's induction curve was constrained at the top to maximum rosiglitazone induction (212-fold induction).

Quantitative PCR analysis

RNA was isolated from differentiating cells in 6-well plates treated as described above on d = 1, d = 2, d = 4, d = 6, and d = 8, using TRIzol reagent (Life Technologies) following manufacturer's instructions. Experiments were repeated three separate times using similar passage number (passage +9 through +13) 3T3-L1 cells. RNA was cleaned and genomic DNA was eliminated by using an RNase-Free DNase Kit (Qiagen) on an RNeasy mini column (Qiagen). RNA (1 μg) was transcribed into cDNA using High Capacity cDNA synthesis kit (Applied Biosystems). Complimentary DNA (25 ng) was amplified in a QuantStudio 7 Flex Real Time PCR System (Applied Biosystems) using Power SYBR Green 2× mastermix (Applied Biosystems) in triplicate. Primer sets for all genes were pre-designed assays from IDT DNA (Redwood City, California) and are as follows: Adipoq Mm.PT.58.29300341, Acaca Mm.PT.58.12492865, Acacb Mm.PT.58.32720646, Cebpa Mm.PT.58.30061639.g, Cebpb Mm.PT.58.6467976.g, Cebpd Mm.PT.58.31539128.g, Esr1 Mm.PT.58.8025728, Dlk1 (Pref1) Mm.PT.58.30309372, Ebf1 Mm.PT.58.30999400, Egr2 (Krox20) Mm.PT.56a.30480551, Esr2 Mm.PT.58.42880809, Esrrg Mm.PT.58.29781526, Fabp4 Mm.PT.58.43866459, Fasn Mm.PT.58.14276063, Fkbp5 Mm.PT.58.45861921, Gpr30 Mm.PT.58.43936136, Insr Mm.PT.58.7145585, Irs1 Mm.PT.58.43919344, Klf4 Mm.PT.58.10779296, Klf5 Mm.PT.58.23426119, Klf15 Mm.PT.56a.31866505, Lep Mm.PT.58.13515402, Lpl Mm.PT.58.45869933, Nr3c1 Mm.PT.58.42952901, Pdk4 Mm.PT.58.9453460, Pparg (detects all variants) Mm.PT.58.10379967, Rpl19 Mm.PT.58.12385796, Rxra Mm.PT.58.42702972, Srebf1 Mm.PT.58.30025542, and Thy1 Mm.PT.58.14029994. Technical replicate Ct values were averaged for each biological replicate. Mean C_t values were normalized to *Rpl19* (60S ribosomal protein L19) (ΔCt), and relative fold change mRNA levels were then calculated by using ΔΔ C_t method (Livak and Schmittgen, 2001) with relative gene expression presented as mean fold change over vehicle control ± SEM ($n = 3$ biological replicates) to visualize the gene expression patterns. Then, the chemical group data was compared with the MDI group for plotting in figures. One-way ANOVA, with multiple comparison Dunnett's *post-hoc* test (GraphPad Prism v.7), were performed on ΔCt values and ΔCt standard error of the mean (SEM) to determine statistical significance between MDI and TBBPA concentrations (·$p < .05$ and ‡$p < .1$) of individual day qPCR data in Figures 4 through 6. Two-way ANOVA, with Dunnett's post-test were performed on ΔCt values and ΔCt SEM to determine statistical significance between MDI and

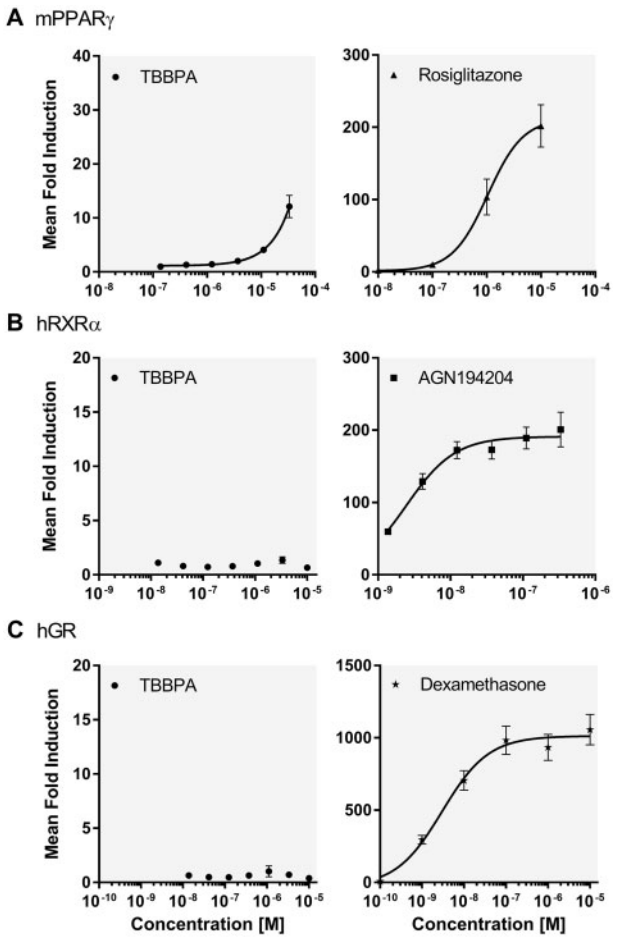

Figure 1. TBBPA and receptor activation assays. Mouse PPARγ receptor activation by (A) TBBPA (AC_{50} = 397 μM) and rosiglitazone (AC_{50} = 1.05 μM). Human RXRα receptor activation by (B) TBBPA and AGN194204 (AC_{50} = 2.45 nM). Human GR activation by (C) TBBPA and dexamethasone (AC_{50} = 3.30 nM). Results are expressed as fold induction over DMSO control per well (mean ± SEM; $n = 3$). Solid black line: nonlinear variable slope curve fit analysis.

TBBPA concentrations (•$p < .05$ and ‡ $p < .1$) for time-course qPCR data in Figure 3.

RESULTS

TBBPA Is a Weak Activator of Mouse PPARγ

The capability of TBBPA to activate the mouse PPARγ receptor was determined using a transient transfection receptor activation assay. As shown in Figure 1A, mouse PPARγ receptor was significantly activated by 10 μM and 33 μM TBBPA, at 4.07 and 12.11-fold induction of luciferase, respectfully, over the 0.05% DMSO controls. Activation of mPPARγ by its full agonist, ROSI, was also evaluated; 33 μM ROSI produced a 200-fold induction over the 0.05% DMSO control. Using a nonlinear regression curve fit analysis with a top constraint set to maximal induction caused by ROSI, TBBPA was assigned an AC_{50} = 397 μM. When compared with the induction levels of ROSI (AC_{50} = 1.05 μM), TBBPA induced only 6% of the total induction aptitude. These data indicate that TBBPA is a weak mPPARγ agonist (Figure 1A). In addition, TBBPA failed to activate the human retinoid X receptor alpha (hRXRα) or the human glucocorticoid receptor (hGR) in a similar assay (Figs. 1B and C).

Induction of 3T3-L1 Adipogenesis and Lipid Accumulation by TBBPA Exposure

Although TBBPA exhibits weak activation of the mouse PPARγ receptor, as shown here, and other species (Riu *et al.*, 2011a), whether TBBPA is directly enhancing adipogenesis or lipid accumulation within the adipocyte, has not been determined. To answer this question, we used low passage mouse 3T3-L1 preadipocytes and high-content quantitative imaging technology to investigate the effects of TBBPA on adipogenesis and lipid accumulation. Total cell counts indicated no signs of cytotoxicity at the concentrations of TBBPA or DMSO tested. High-content microscopy images illustrated that 10 μM TBBPA alone visually increased the number of adipocytes, as judged by the presence of morphological adipocytes; rounded cells with increased abundance of lipid droplets (Figure 2A). In addition,

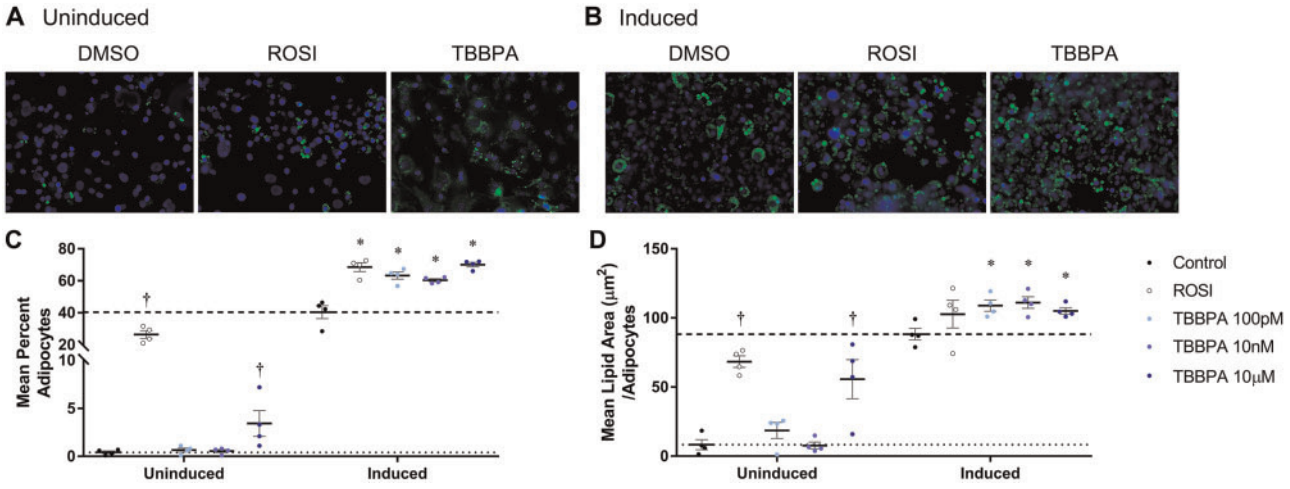

Figure 2. TBBPA effects on adipocyte differentiation and lipid accumulation in 8 day assays. 3T3-L1 cells were either grown in adipocyte maintenance media (uninduced) (A) or treated (induced) with MDI (B) for 8 days with and without TBBPA. DMSO: vehicle control, ROSI: 1 μM rosiglitazone positive control, and 10 μM TBBPA. Cells were co-stained with Hoechst (nuclei) and Nile red (lipid). High content capture of images using DAPI and FITC filters provided data on (C) mean percent adipocytes per well and (D) mean lipid area (μm²) per adipocyte per well (mean ± SEM, $n = 4$). Dash line: induced control mean. Dotted line: uninduced control mean. One-way ANOVA with multiple comparisons Dunnett's post hoc test $p < .05$, • statistically significant over induced control, $p < .05$, and † statistically significant over uninduced vehicle control, $p < .05$.

10 μM TBBPA treatment with MDI induced a visual increase in percent of adipocytes over that of MDI alone (Figure 2B). High-content images were used to quantify the number of mature adipocytes (percent adipocytes; Figure 2C) and the amount of lipid accumulating (μm^2) per adipocyte (Figure 2D). TBBPA (10 μM) alone initiated a significant increase in both the number of cells becoming adipocytes and lipid accumulation compared with vehicle control cells (uninduced). Co-treatment of TBBPA (100 pM, 10 nM, and 10 μM) with MDI (induced control) increased both adipocyte number and lipid droplet accumulation over that of MDI alone. ROSI, with or without MDI, increased the number of adipocytes and the amount of accumulating lipid over the respective controls, as expected. Taken together, these data suggest that adipogenic and lipogenic responses are triggered by exposure to 10 μM TBBPA alone, and that low concentrations of TBBPA (100 pM) are approximately as effective as 1 μM ROSI in stimulating a robust adipogenic response in combination with MDI.

TBBPA Induced Precocious mRNA Expression Pattern of Cebpa and Pparg

To investigate the timing and mechanism for TBBPA induced adipogenesis, 3T3-L1 preadipocytes were differentiated in the presence of several concentrations of TBBPA and RNA was collected on 1, 2, 4, 6, and 8 days post induction. By 4 days post MDI, 10 μM TBBPA and ROSI had significantly elevated expression of both *Cebpa* and *Pparg* compared to MDI alone (Figs. 3A and B). These effects remained apparent on day 6, and by day 8 all concentrations of TBBPA had increased expression of these 2 genes compared with MDI controls (Figs. 3A and B). In addition, we evaluated numerous other adipocyte-specific genes after 8 days of TBBPA treatment and found that not only *Cebpa* and *Pparg* were upregulated, but most concentrations of TBBPA tested induced robust increases in *Fabp4*, adiponectin (*Adipoq*), and leptin (*Lep*) (Figs. 4A, C, and D and Supplementary Figure 1). Significant effects of 3 out of 4 concentrations of TBBPA were also seen in acetyl-CoA carboxylase-alpha (*Acaca*), acetyl-CoA carboxylase-beta (*Acacb*), fatty acid synthase (*Fasn*), and lipoprotein lipase (*Lpl*) (Figs. 4A and E). Effects of the highest concentration of TBBPA also significantly upregulated *Rxra* above that of MDI positive controls (Figure 4B). At no point did the effects of high concentrations of TBBPA exceed that of ROSI, a full PPARγ agonist, but in nearly all cases the gene expression stimulated by TBBPA was within a factor of 2 from the ROSI-induced gene expression. These data suggest partial agonism of PPARγ by TBBPA and the precocious induction of *Cebpa* and *Pparg* above that of MDI indicate that TBBPA affects an earlier receptor or transcription factor in the differentiation pathway.

TBBPA Affects Early Adipogenesis Transcription Factors

C/EBPβ and C/EBPδ are known upstream regulators of C/EBPα and PPARγ (Cao *et al.*, 1991; Rosen and MacDougald, 2006), and are transcriptionally activated during the early phase of differentiation, with peak mRNA expression observed 48 h after induction (Supplementary Figure 1). When exposed to some concentrations of TBBPA for only 2 days, the 3T3-L1 cells increased mRNA levels of *Cebpd* and sterol regulatory element binding factor-1 (*Srebf1*), a transcription factor required for sterol biosynthesis and lipid homeostasis (Figure 5A). However, there was no significant change in expression of either *Cebpb* or its upstream activator *Krox20* (Figs. 5A and C) with TBBPA exposure.

Transcriptional activation of *Cebpd* has been linked to glucocorticoid signaling, and is directly responsive to hormones present in the adipogenic media, mainly dexamethasone (Cao *et al.*, 1991). To explore this linkage, we analyzed the mRNA expression of the GR (*Nr3c1*), pyruvate dehydrogenase kinase (*Pdk4*, a GR downstream gene), and FK506-binding protein-5 (*Fkbp5*, a co-regulator of GR). Low concentrations of TBBPA increased GR mRNA levels compared with MDI controls on day 8 (Figure 6C), but effects at higher concentrations did not reach significance; however, levels of *Pdk4* were significantly increased at the 10 μM TBBPA level ($p < .05$) (Figure 6C). *Fkbp5* expression pattern was similar to *Cebpd*, with maximal expression on day 2 post induction, and was significantly increased by most TBBPA exposures (Figure 5B; Supplementary Figure 1).

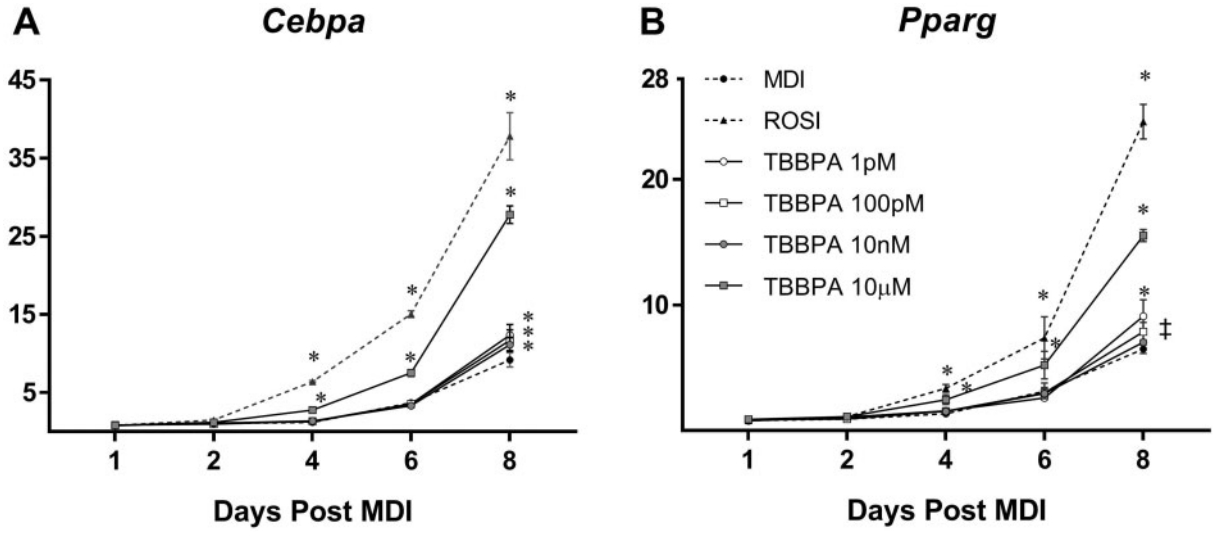

Figure 3. Critical adipogenic transcription factor mRNA expression profiles during differentiation. Two day post-confluent 3T3-L1 cells were treated for 8 days with induction agents (MDI) and exposed to TBBPA. MDI: adipogenesis positive control, ROSI: rosiglitazone and MDI (PPARγ agonist positive control). Messenger RNA expression was quantified by RT-qPCR (A) *Cebpa* mRNA levels (B) *Pparg* mRNA levels. Relative gene expression data are presented as mean fold change over vehicle control ± SEM, $n = 3$. Two-way ANOVA with multiple comparisons Dunnett's *post-hoc* test was performed on ΔCt data, * statistically significant over MDI control, $p < .05$. ‡ statistically significant over MDI control, $p < .1$ (*Pparg*, day 8, 10 nM, TBBPA, $p = .06$).

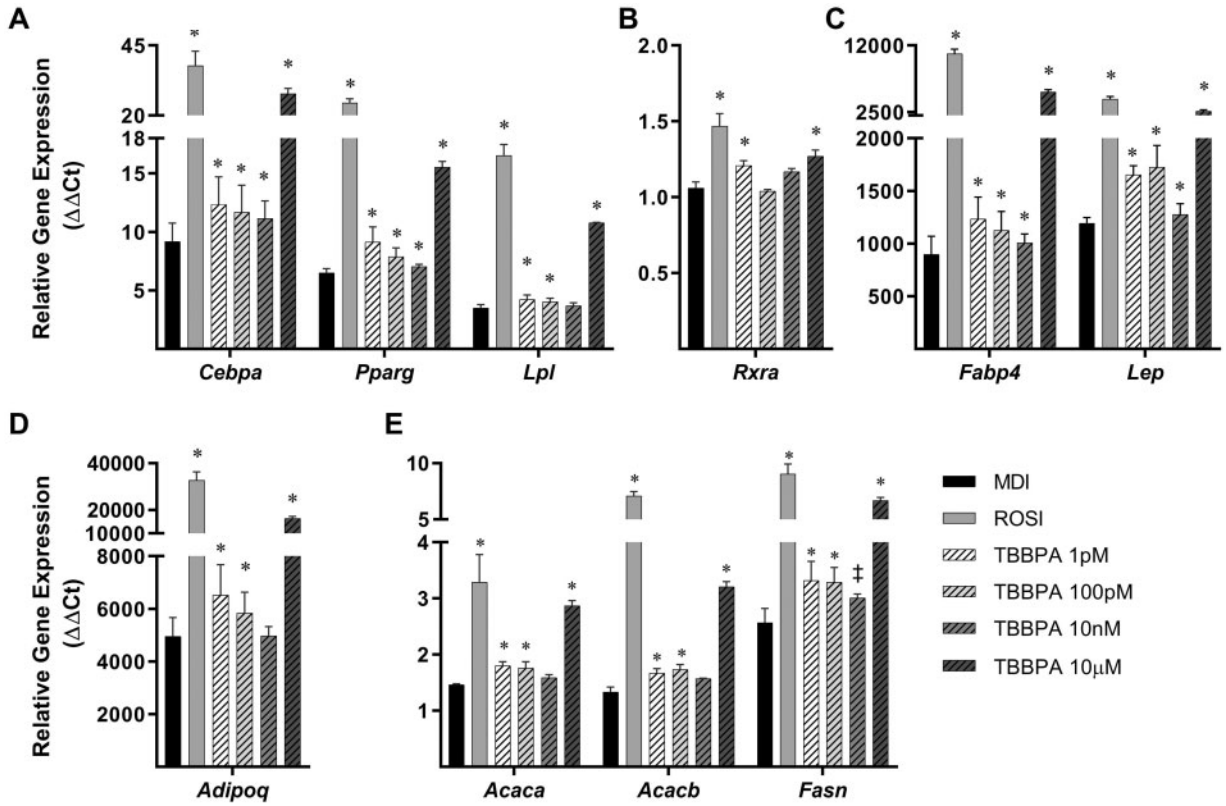

Figure 4. TBBPA effects on adipogenesis and adipocyte-specific mRNAs by day 8 of differentiation. Two-day post-confluent 3T3-L1 cells were treated for 2 days with induction agents (MDI) and TBBPA for 8 days. MDI: adipogenesis positive control, ROSI: rosiglitazone and MDI (PPARγ agonist positive control). Messenger RNA expression was quantified by RT-qPCR for (A and B) adipogenesis genes, (C and D) adipocyte-specific genes, and (E) lipogenic genes. Relative gene expression data are presented as mean fold change over vehicle control ± SEM, $n = 3$. One-way ANOVA with multiple comparisons Dunnett's *post-hoc* was performed on ΔCt data, * statistically significant over MDI control, $p < .05$. ‡ statistically significant over MDI control, $p < .1$ (*Fasn*, 10 nM TBBPA, $p = .052$).

The maximally increased expression of *Cebpd*, *Srebf1*, *and Fkbp5* on day 2 of differentiation illustrates that TBBPA is accentuating early genes in differentiation, prior to PPARγ expression and activation, in a specific (*Cebpd*) part of the pathway.

While most studies have focused on the role of PPARs and C/EBPs in adipocyte differentiation, it is known that many other transcription factors and co-factors are involved prior to the expression of the major regulators of terminal differentiation. These include Krüppel–like factors (*Klf4*, *Klf5*, and *Klf15*), zinc-finger-containing factor (*Krox20*), and an early B-cell factor (*Ebf1*) (Jimenez *et al.*, 2007; Wu and Wang, 2013). On day 2 of differentiation, TBBPA exposure had not affected *Klf4* mRNA expression, compared with the MDI control group (Figure 5D). However, 10 μM TBBPA increased *Klf15* over MDI controls on day 2 (Figure 5D), and thereafter was significantly increased by all TBBPA concentrations tested on day 8 (Figures 5E; Supplementary Figure 1). Klf5 was also increased by day 2 of culture following 10 μM TBBPA treatment, but *Ebf1* expression did not significantly change throughout the 8 day differentiation process (Figure 5C; Supplementary Figure 1).

As adipocyte genes are starting to be expressed, preadipocyte genes are being repressed. We also analyzed mRNA expression of two such preadipocyte genes whose repression are required before adipogenesis proceeds. First, the preadipocyte factor-1 (*Pref1*) mRNA expression was significantly decreased at 100 pM TBBPA group on day 2 of differentiation (Figure 5C), an effect not seen in the ROSI group. Secondly, *Thy1* (CD90) mRNA expression was recently shown by Flores *et al.*, to decrease upon treatment with TBBPA and therefore increase adipogenic potential of 3T3-L1 cells (Flores and Phipps, 2014; Woeller *et al.*, 2017). Concurrently, we saw a significant decrease in *Thy1* expression when exposed to the highest concentration of TBBPA or ROSI on day 2 of differentiation and this downregulation continued through day 8 of TBBPA exposure (Figure 5C, Supplementary Figure 1).

TBBPA Impact on Nontraditional Estrogen Receptor Gene Expression

Despite sharing structural similarity to estrogens, several studies have shown that TBBPA lacks estrogen-like effects *in vivo* (ECB, 2008; Lai *et al.*, 2015) and lacks the capability to bind to the estrogen receptor alpha (ERα) *in vitro* (Riu *et al.*, 2011b). Nonetheless, TBBPA may affect gene expression of estrogen receptors or the estrogen-related receptors and affect estrogen signaling pathways in another manner than a direct ERα binding. Indeed, estrogen-related receptor gamma (*Esrrg*) mRNA was increased with 100 pM TBBPA on day 8 of differentiation, but the G-coupled protein receptor-30 (*Gpr30*) was significantly increased over MDI control at all concentrations of TBBPA tested (Figure 6B); 1 pM and 10 μM TBBPA also increased expression of insulin receptor (*Insr*) and its adaptor protein (*Irs1*) (100 pM and 10 μM TBBPA) above the level seen in MDI controls (Figure 6D). Estrogen receptor alpha (*Esr1*) was down-regulated to a significantly lesser extent than MDI controls at 1 pM, and estrogen receptor beta (*Esr2*) mRNA levels were significantly reduced by

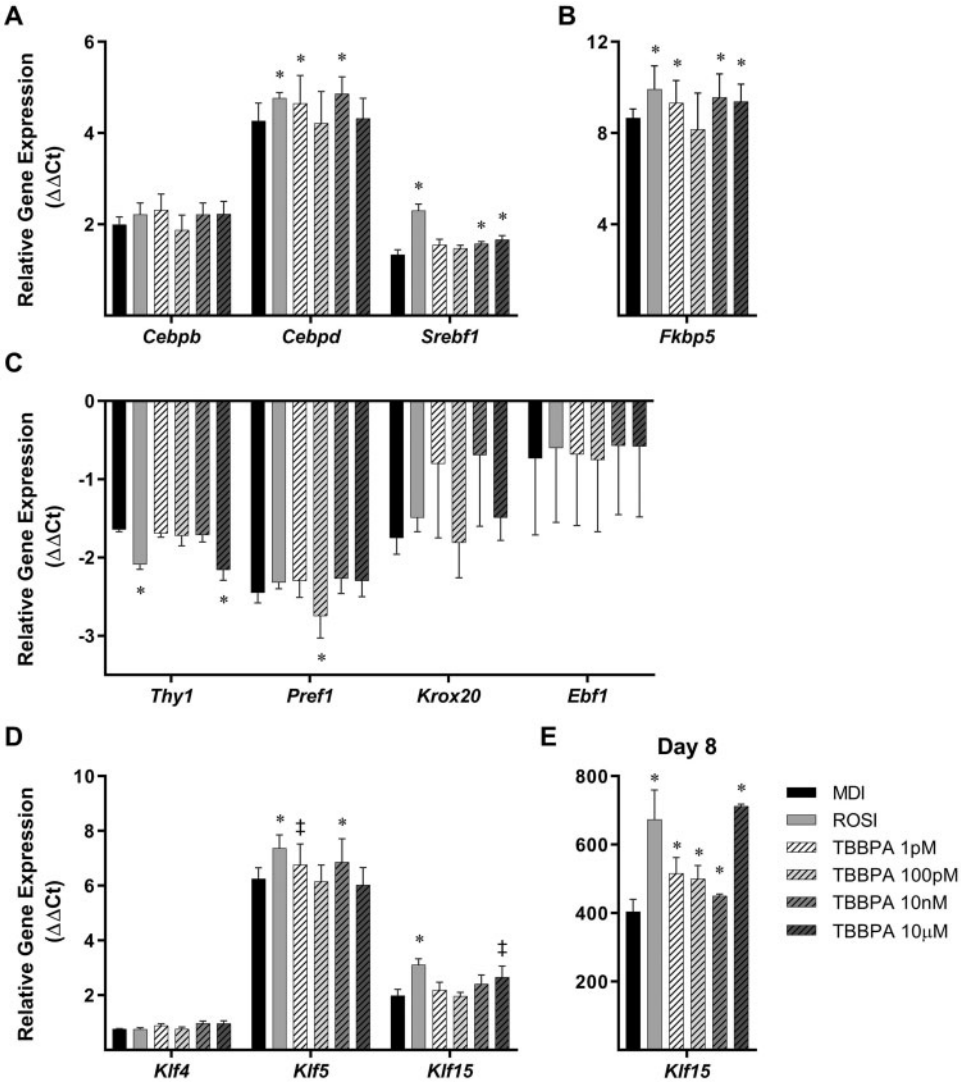

Figure 5. Preadipocyte and adipogenic mRNAs altered early in differentiation by TBBPA. Two-day post-confluent 3T3-L1 cells were treated for 2 days with induction agents (MDI) and TBBPA. MDI: adipogenesis positive control, ROSI: rosiglitazone and MDI (PPARγ agonist positive control). Messenger RNA expression was quantified by RT-qPCR. (A and B) Early adipogenesis transcription factors on day 2, (C) preadipocyte genes on day 2, (D) selected Krüppel-like factors on day 2, and (E) Klf15 on day 8 of the differentiation assay. Relative gene expression data are presented as mean fold change over vehicle control ± SEM, $n = 3$. One-way ANOVA with multiple comparisons Dunnett's *post-hoc* test was performed on ΔCt data, * statistically significant over MDI control, $p < .05$. ‡ statistically significant over MDI control, $p < .1$ (*Klf5*, 1 pM TBBPA, $p = .08$, *Klf15*, 10 μM TBBPA, $p = .08$).

ROSI and 10 μM TBBPA treatment (Figure 6A). Conversely, 100 pM and 10 nM TBBPA increased Esr2 levels. These data suggest that TBBPA may affect estrogen signaling in the mature adipocyte, however, not through the binding or activation of traditional estrogen receptors. Further studies are needed to clarify the role that these receptors, especially *Gpr30*, may play in TBBPA-mediated effects on adipocyte differentiation.

TBBPA Pre-Treatment Enhances Differentiation of 3T3-L1 Cells

In Figure 2, we demonstrate an escalation in both adipocyte number and lipid accumulation of 3T3-L1 adipocytes when exposed to TBBPA during the differentiation process. In addition, our data suggests a TBBPA-specific decrease in preadipocyte genes early in differentiation (Figure 5C) and a precocious increase in critical adipogenesis genes (Figs. 5A, B, and D) when co-exposed to TBBPA and MDI. This led us to postulate that TBBPA might increase the preadipocytes efficiency of differentiation into adipocytes. We exposed *proliferating* 3T3-L1 cells to TBBPA and induced them to differentiate. High-content microscopy data illustrated that cells pre-treated with TBBPA then induced to differentiate showed significant increases in adipocyte number and lipid accumulation over MDI control cells (Figs. 7A, induced and B, induced). This same effect was seen for several TBBPA concentrations in the uninduced cells (Figs. 7A, uninduced and B, uninduced). These data suggest that TBBPA could influence genes required for the preadipocyte-to-adipocyte transition, which is consistent with the enhanced early gene expression seen on day 2 in these studies. The mechanism(s) by which TBBPA accelerates or primes the differentiation process during the early phases is not completely clear, but likely involves more than one specific pathway/factor.

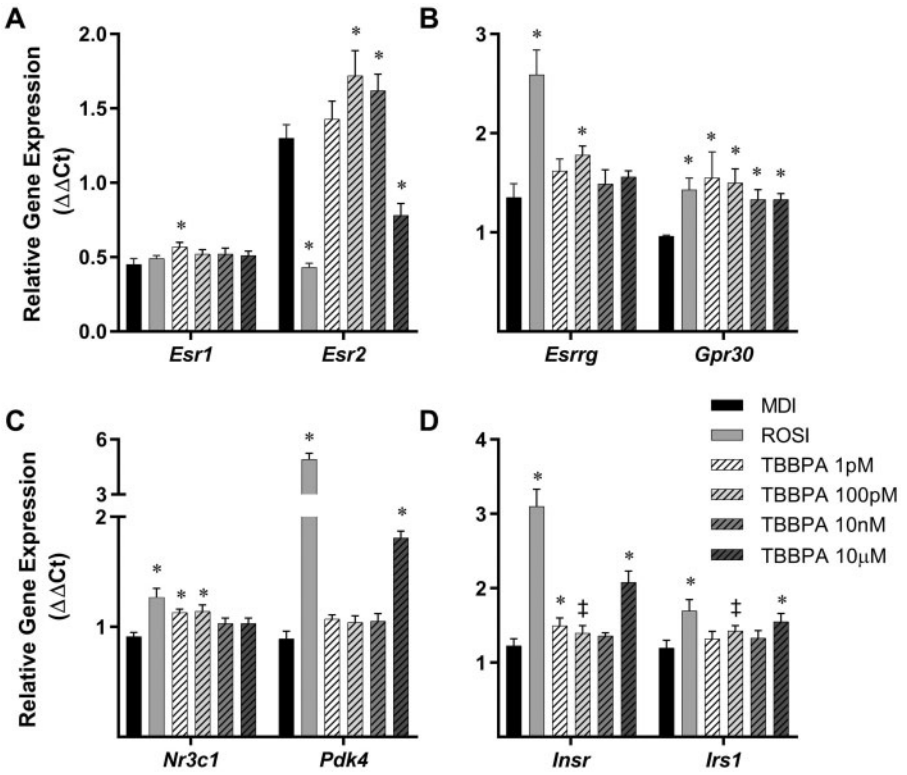

Figure 6. Receptor mRNA levels 8 days after induction and TBBPA exposure. Two-day post-confluent 3T3-L1 cells were treated for 2 days with induction agents MDI and exposed to TBBPA for 8 days. MDI: adipogenesis positive control, ROSI: rosiglitazone and MDI (PPARγ agonist positive control). Messenger RNA expression was quantified by RT-qPCR for (A) estrogen receptors -alpha and -beta, (B) estrogen-related receptors, (C) GR (*Nr3c1*) and a downstream gene (*Pdk4*), and (D) insulin receptors and insulin receptor substrate-1. Relative gene expression data are presented as mean fold change over vehicle control ± SEM, $n = 3$. One-way ANOVA with multiple comparisons Dunnett's *post-hoc* test was performed on ΔCt data, ⋆ statistically significant over MDI control, $p < .05$. ‡ statistically significant over MDI control, $p < .1$ (*Insr*, 100 pM TBBPA, $p = .1$, *Irs1*, 100 pM TBBPA, $p = .06$).

DISCUSSION

In the present study, the goal was to delineate the mechanistic pathway used by TBBPA during differentiation of adipocytes. We accomplished this goal using low passage 3T3-L1 mouse preadipocytes, in which we observed the temporal changes in gene expression regulated during TBBPA-induced adipogenesis. TBBPA significantly increased adipocyte number, lipid accumulation, and caused early expression of adipocyte genes needed for terminal differentiation. Unlike previous results demonstrating PPARγ as a target for TBBPA-induced adipogenesis (Riu *et al.*, 2011a), these data suggest that TBBPA (in the presence of differentiation media) affects differentiation within the first 48 h of the process by both quickly decreasing preadipocyte genes, such as *Thy1* and possibly *Pref1*, and increasing key early adipogenic genes that together enhance PPARγ expression (C/EBPδ, SREBF1, FKBP5, KLF15) and dramatically increased expression of other key factors (C/EBPα, FABP4, LEP, and ADIPOQ) shortly thereafter. We also demonstrated novel effects of TBBPA, in the absence of MDI; TBBPA caused an increased percentage of proliferating preadipocytes to commit to becoming adipocytes.

These findings, and the fact that ROSI affected primarily PPARγ down-stream gene targets and not the TBBPA-effected early genes, suggest that novel modes of action exist for TBBPA-induced adipogenesis and lipid accumulation.

Regulation of PPARγ Signaling Pathway by TBBPA

Previous studies have discovered that halogenated bisphenols, including TBBPA, are activators of human, zebrafish and Xenopus PPARγ (Akiyama *et al.*, 2015; Riu *et al.*, 2011a). Timing is critical to adipogenesis, which is a highly regulated process of temporal transcription factor expression. Among the transcription factors involved in this cascade, C/EBPα and PPARγ have been shown to regulate each other's transcription through a positive feedback loop and are absolutely critical to the progression of adipogenesis, the expression of terminal adipocyte-specific genes, and insulin sensitivity (Rosen *et al.*, 2002; Wu *et al.*, 1999). Previous studies have shown TBBPA can increase mRNA levels of both C/EBPα and PPARγ in 3T3-L1 adipocytes at the end of differentiation at days 8 – 14 after induction (Akiyama *et al.*, 2015; Riu *et al.*, 2011a). The PPARγ amino acid sequence differs between species and because the mouse is our chosen *in vivo* and *in vitro* model species, we tested TBBPA's ability to activate mouse PPARγ. TBBPA was a weak activator of mouse PPARγ, with an AC_{50} of 397 μM. This is in stark contrast to pharmaceuticals such as ROSI (1.05 μM for mPPARγ, Figure 1), or other environmental chemicals such as tributyltin [0.19 μM for mPPARγ, (Li *et al.*, 2011)].

Several transcriptional regulators drive the conversion of preadipocytes into adipocytes; these include members of the C/EBP and KLF families and SREBF1 (Figure 8). The expression of these factors is often rapid, transient, and sequential in order throughout differentiation. Upon hormonal stimulation, expression of early growth factor-responsive transcription factors, like c-myc, c-fos, and c-jun is followed by the short-lived expression of *Krox20* and *Klf4*. These events rapidly upregulate *Cebpd* and *Cebpb*, which induces *Cebpa* and subsequently *Pparg*, which are considered the master regulators of adipogenesis [reviewed in Farmer (2005), Hamm *et al.* (2001); Rosen and MacDougald,

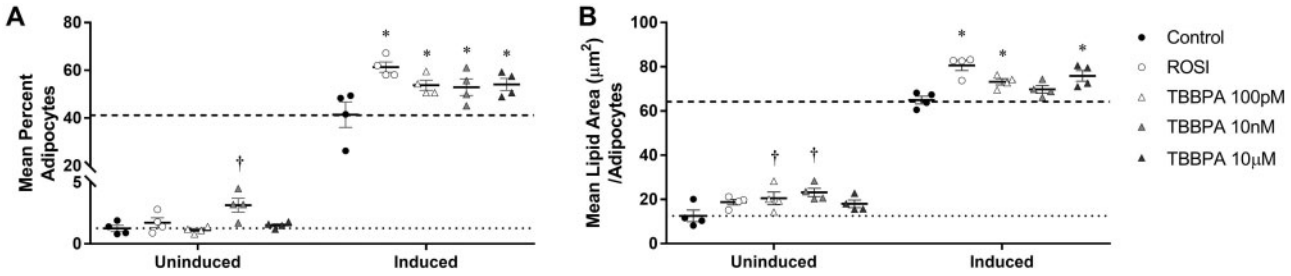

Figure 7. Effects of TBBPA exposure during 3T3-L1 cell proliferation on adipogenesis and lipid accumulation. 3T3-L1 cells were treated with TBBPA during cell proliferation prior to monolayer confluence and then induced to differentiate two days later. High content imaging analysis of cells provided (A) mean percent adipocytes per well and (B) mean lipid area (μm^2) per adipocyte per well (mean ± SEM, $n = 4$). Dash line: induced control mean. Dotted line: uninduced control mean. Two-way AVOVA with multiple comparisons Dunnett's *post-hoc* test, * statistically significant over induced control, $p < .05$, and † statistically significant over uninduced vehicle control, $p < .05$.

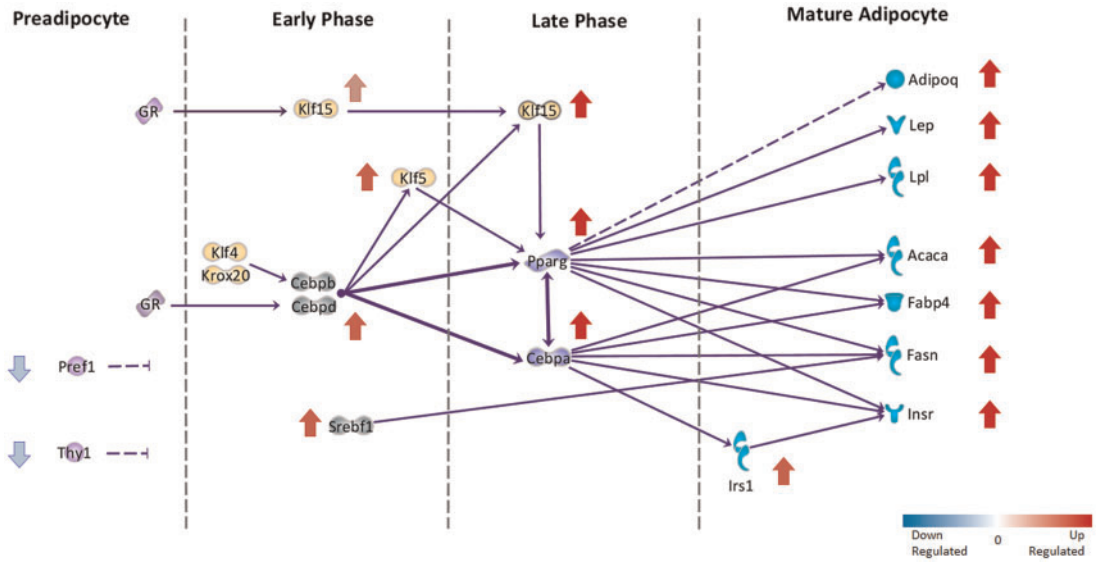

Figure 8. Regulators of adipogenesis and adipocyte-specific genes. Transcription factors and NRs involved in early and late phases of differentiation; mature adipocyte-specific genes and their upstream regulators. Solid lines denote reported direct interactions. Dashed lines indicate proposed indirect interactions. Color-coded up and down arrows indicate TBBPA-induced or -repressed effects on gene expression. Image created using Ingenuity Pathway Analysis software version: #31813283. Adapted from Wu *et al.* (2013).

(2006)]. PPARγ has two main protein isoforms (PPARγ1 and PPARγ2), PPARγ2 is the predominate isoform in adipose tissue, whereas PPARγ1 is expressed at lower levels in adipose as well as in other cell types; both PPARγ1 and PPARγ2 are expressed upon induction of adipogenesis (Rosen and MacDougald, 2006; Tyagi *et al.*, 2011).

C/EBPα and PPARγ have been shown to regulate each other's transcription through a positive feedback loop and are absolutely critical to the progression of adipogenesis, the expression of terminal adipocyte-specific genes, and insulin sensitivity (Rosen *et al.*, 2002; Wu *et al.*, 1999). As protein expression of C/EBPα and PPARγ rise to maximal levels, expression of C/EBPβ and C/EBPδ diminishes. Another transcription factor, SREBF1, regulates genes required for fatty acid and lipid production, and is also expressed early in the differentiation process and is regulated by insulin (Rosen and MacDougald, 2006; Wu *et al.*, 1999). Previous studies have shown TBBPA can increase mRNA levels of both C/EBPα and PPARγ in 3T3-L1 adipocytes at the end of differentiation at days 8–14 after induction (Akiyama *et al.*, 2015; Riu *et al.*, 2011a).

In these studies, not only did TBBPA increase mRNA expression of two important regulators of adipogenesis (*Cebpa* and *Pparg;* Figure 3), but also increased mRNA levels of genes expressed in mature adipocytes, such as *Insr*, *Irs1*, *Lep*, *Adipoq*, *Lpl*, *Fasn*, *Fabp4*, *Acaca*, and *Acacb* at numerous concentrations tested (Figs. 4 and 6). The exact mechanism initiating this series of upregulated genes was not clear, so it was critical to evaluate earlier events following TBBPA exposure. Upstream factors involved in the early phases of adipogenesis (*Cebpd*, *Srebf1*, *Fkbp5*, *Klf5*, and *Klf15*) were significantly upregulated after 2 days of TBBPA treatment. Taking all of these changes together, TBBPA appeared to advance the adipocyte differentiation time course. Numerous genes involved in the early phase of differentiation (see Figure 8) were significantly increased by TBBPA (10 μM) above MDI controls early in the 8 day experiments and were often expressed at the levels induced by ROSI, but sometimes by lower TBBPA concentrations (ie, *Fkbp5 and Cebpd;* Figs. 5A and B, Supplementary Figure 1).

Several Krüppel-like factors are expressed in adipose tissue and have been shown to regulate apoptosis, proliferation, and differentiation (Figure 8). Due to their involvement with early phase differentiation, three KLF's were investigated for TBBPA-induced effects on mRNA expression within 48 h of induction. KLF4 transactivates *Cebpb* by directly binding to the promoter

region and has been shown to act as a co-activator along with KROX20 (Birsoy *et al.*, 2008; Chen *et al.*, 2005). *Klf5* expression is induced by the heterodimer duo of C/EBPβ and C/EBPδ, which may bind to its promoter. In late phase differentiation, KLF5 binds and activates the *Pparg2* promoter (Oishi *et al.*, 2005). *Klf15* has been identified as a target of GR during adipogenesis (Asada *et al.*, 2011). Asada *et al.*, further showed that GR binds directly to GRE sites on the promoter of *Klf15*, and that this interaction can occur rapidly after 3T3-L1s stimulation with dexamethasone (Asada *et al.*, 2011). In addition, *Klf15* expression is maintained during early phase adipogenesis by C/EBPβ and C/EBPδ, then as its expression rises during late phase differentiation, KLF15 can increase the expression of PPARγ, although binding sites have not yet been discovered (Asada *et al.*, 2011; Wu and Wang, 2013). TBBPA exposure increased *Klf5*, *Klf15*, and *Cebpd* mRNA expression early in differentiation, and had no effect on the *Klf4*, *Krox20*, *Cebpb* signaling network. Considering the timing of adipogenesis, these data suggest that TBBPA is affecting *Cebpd*, *Klf15*, *and Klf5* prior to day 2 of differentiation, which may lead to the earlier expression and/or increased transactivation of both *Pparg* and *Cebpa* in the late phase of differentiation. Although we did not test for this, future studies may investigate the role of these TBBPA-induced genes specifically on the *Pparg2* promoter.

Potential Role of GR in TBBPA-Induced Adipogenesis and Lipid Accumulation

As stated, regulation of both *Klf15* and *Cebpd* has been linked to glucocorticoid signaling during adipogenesis (Asada *et al.*, 2011). Glucocorticoids may induce adipogenesis *in vitro*; however, the role of the GR in *in vivo* adipogenesis is still poorly understood (Cao *et al.*, 1991). Upon ligand binding, GR translocates from the cytosol to the nucleus where it regulates gene transcription. GR activation and translocation is regulated by a multiprotein HSP90/HSP70-based chaperone complex important for proper folding, maturation, and trafficking. FKBP5 is a co-chaperone protein that, when bound to the larger chaperone complex, regulates GR sensitivity to ligands and retains GR in the cytosol (Binder, 2009; Pratt *et al.*, 2006). *Fkbp5* mRNA expression is increased by glucocorticoids setting off a negative feedback loop, where FKBP5 then binds and regulates GR sensitivity to further stimulation by ligands (Vermeer *et al.*, 2003). Interestingly, we showed a TBBPA-induced increase in *Fkbp5* mRNA by day 2 of differentiation, suggesting a GR ligand-stimulated increase in *Fkbp5* over that of the MDI control that already contains low levels of dexamethasone. Previous studies have shown that other EDCs can mimic GR signaling by competitive binding. Sargis *et al.* demonstrated that BPA can significantly stimulate GR-mediated reporter expression (Sargis *et al.*, 2010). Also, a recent study has shown that a PPARγ antagonist only partially inhibited TBBPA-induced adipogenesis suggesting other important pathways were involved (Riu *et al.*, 2011a). Our studies demonstrated TBBPA-stimulated early upregulation of genes known to be involved in GR-mediated adipocyte differentiation (*Klf15*, *Fkbp5*, *Cebpd*), and *Pdk4* (high concentrations only), a downstream gene regulated by GR activation, but the receptor activation assay for TBBPA-induced human GR activation was negative (Figure 1C). Although we also demonstrate limited evidence of GR mRNA expression increases at low concentrations of TBBPA (*Nrc3c1*), these data do not provide strong evidence for a primary role of the GR in TBBPA-stimulated effects, but rather that the effect of TBBPA are likely mediated through a synergism of multiple pathways, likely a noncanonical GR pathway or another growth factor pathway that synergizes with PPARγ signaling that has not yet been characterized. Future work using specific inhibitors may help identify the key missing elements.

Novel Mechanisms Involved in TBBPA Effects in 3T3-L1 Cells

Promptly after differentiation induction, preadipocyte genes must be repressed before expression of adipocyte genes are initiated. The most well-known, preadipocyte factor-1 (*Pref1*), is highly expressed in the preadipocyte and its active form inhibits adipogenesis presumably through interaction with Notch (Rosen and MacDougald, 2006; Rosen and Spiegelman, 2014). *Pref1* mRNA expression was significantly decreased by TBBPA (100 pM) treatment on day 2 after induction. *Pref1* levels remained repressed throughout the remainder of differentiation in both MDI and TBBPA groups compared with the vehicle control (Figure 5C and Supplementary Figure 1). It is a possibility that 1–2 days post induction was too late to catch the repression of *Pref-1*; further investigation is warranted. *Thy1* (CD90) is a cell surface protein belonging to the immunoglobulin superfamily and is expressed in many cell types, including fibroblasts and stem cells. In 3T3-L1 cells, *Thy1* expression hampers PPARγ activity through inhibition of the Src family kinase, *Fyn* (Woeller *et al.*, 2015). It was recently shown in 3T3-L1 cells that TBBPA (1 and 5 μM) exposure for 8 days in culture decreases *Thy1* mRNA expression and therefore increased adipogenic potential of 3T3-L1 cells (Flores and Phipps, 2014; Woeller *et al.*, 2017). Concurrently, a decrease of *Thy1* expression following high level TBBPA exposure was seen in our studies on day 2 of differentiation. However, a similar significant decrease in *Thy1* expression with treatment with rosiglitazone was noted suggesting that this is not a TBBPA-specific early event. The fact that both *Pref1* and *Thy1* were decreased by some, but not all TBBPA exposures suggested consistency with our hypothesis; more than one signaling pathway is needed for TBBPA-induced adipogenesis. Also, we may have missed the peak in repression of these genes by TBBPA as our studies weren't designed to examine these early gene events.

Members of the NR superfamily have been historic targets of EDCs (Heindel *et al.*, 2017; Janesick and Blumberg, 2011). Two of these NRs have been implicated in this study as targets of TBBPA already (GR and PPARγ). In addition to these, sex steroid receptors have also been connected to EDC mode of actions (estrogen, progesterone, and thyroid receptors). Adipose tissue is a target for sex steroids, as their receptors are expressed in rodent and human adipocytes. Both PPARγ and ERs are members of the same NR superfamily and share similar co-factors/co-regulators. In the current study, TBBPA diminished the MDI-induced inhibition of *Esr1*and *Esr2* at low concentrations and enhanced the repression of *Esr2* mRNA expression at the highest concentration tested (similar to that seen by ROSI), suggesting varied effects on ERs by exposure level. Given those results, the effects of TBBPA on expression of other members of the estrogen receptor family were investigated in these studies. TBBPA treatment exacerbated *Gpr30* expression during differentiation at all concentrations tested, with effects equivalent to that of ROSI. Recently, the G-coupled protein receptor (*Gpr30*, a membrane-bound ER), was linked to obesity and diabetes (Sharma and Prossnitz, 2011; Sharma *et al.*, 2013). *Gpr30* has a high affinity for 17 β-estradiol and is expressed in numerous tissues, including adipose tissue. Obese female mice have elevated levels of *Gpr30* mRNA in their white adipose tissue and GPR30 knockout female mice were protected from high-fat diet induced obesity (Wang *et al.*, 2016). Likewise, GPR30 has been

shown to enhance adipogenesis of 3T3-L1 preadipocytes when activated by G1, a specific GPR30 agonist (Wang *et al.*, 2016). Interestingly, multiple reports have shown that GPR30 and ERα inhibit each other's actions. Consistent with those reports, both *Esr1* and *Esr2* expression was repressed in the positive control (MDI) and *Gpr30* was induced during early differentiation (Supplementary Figure 1) in these studies. It is yet to be determined what type of interaction TBBPA exerts on GPR30; and whether direct or indirect activation is evident at the protein level.

Estrogen-related receptors (*Esrra* and *Esrrg*) play a novel role in regulating adipocyte gene expression through interactions with PGC1a, a PPARγ co-activator (Huss *et al.*, 2002; Ijichi *et al.*, 2007). It has been shown that BPA can positively regulate the activity of human ERRγ by binding directly with the receptor (Matsushima *et al.*, 2007; Takayanagi *et al.*, 2006). Currently there are no studies showing TBBPA binding or activating ERRs directly, however, TBBPA significantly induced expression of *Esrrg* mRNA above that of the MDI control group but did not approach the level of gene expression induced by ROSI (Figure 6B and Supplementary Figure 1).

In these studies, we presumed that the numerous, time- and dose-dependent changes in RNA expression were causative. Future experiments should evaluate the protein and/or activity of the gene products in these implicated pathways to confirm our theory. Additionally, future studies would benefit from incorporation of inhibitors or antagonists of specific receptors whose RNA were significantly changed to help determine the primary target for TBBPA.

CONCLUSIONS

We provide evidence of TBBPA-induced early gene expression changes in the GR, PPARγ, and nontraditional ER pathways, leading to promotion of adipocyte differentiation. Several of these gene targets are novel, not recapitulated by ROSI (strict PPARγ agonist), and sensitive to low concentrations of TBBPA. Taken together, these data demonstrate that TBBPA is affecting NR signaling during various stages of adipocyte differentiation; each contributing to increased adipocyte number and accumulation of lipids within those adipocytes. TBBPA also induced down regulation of critical preadipocyte genes (*Pref-1* and *Thy1*) known to inhibit differentiation and could commit cells to mature adipocytes without further treatments. These studies suggest early genes, such as *Thy1*, *Cebpd*, *Fkbp5*, *Kfl15*, and *Srebf1* are important targets for TBBPA-induced adipogenesis that eventually includes enhanced PPARγ and GR pathways.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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