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Tributyltin Differentially Promotes Development of a Phenotypically Distinct Adipocyte

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Abstract

Objective—Environmental endocrine disrupting chemicals (EDCs) are increasingly implicated in the pathogenesis of obesity. Evidence implicates various EDCs as being pro-adipogenic, including tributyltin (TBT), which activates the peroxisome proliferator activated receptor- γ (PPAR γ). However, the conditions required for TBT-induced adipogenesis and its functional consequences are incompletely known.

Methods—The co-stimulatory conditions necessary for preadipocyte-to-adipocyte differentiation were compared between TBT and the pharmacological PPAR γ agonist troglitazone (Trog) in the 3T3-L1 cell line; basal and insulin-stimulated glucose uptake were assessed using radiolabeled 2-deoxyglucose.

Results—TBT enhanced expression of the adipocyte marker C/EBP α with co-exposure to either isobutylmethylxanthine or insulin in the absence of other adipogenic stimuli. Examination of several adipocyte-specific proteins revealed that TBT and Trog differentially affected protein expression despite comparable PPAR γ stimulation. In particular, TBT reduced adiponectin expression upon maximal adipogenic stimulation. Under submaximal stimulation, TBT and Trog differentially promoted adipocyte-specific gene expression despite similar lipid accumulation. Moreover, TBT attenuated Trog-induced adipocyte gene expression under conditions of co-treatment. Finally, TBT-induced adipocytes exhibited altered glucose metabolism, with increased basal glucose uptake.

Conclusions—TBT-induced adipocytes are functionally distinct from those generated by a pharmacological PPAR γ agonist, suggesting that obesogen-induced adipogenesis may generate dysfunctional adipocytes with the capacity to deleteriously affect global energy homeostasis.

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Keywords

adipocyte; adipogenesis; adiponectin; endocrine disrupting chemicals; PPAR γ ; tributyltin; troglitazone

Introduction

Global metabolic health has deteriorated dramatically over the last several decades with the emergence of the dual obesity and diabetes epidemics. While increased caloric consumption and physical inactivity are central drivers in the pathogenesis of metabolic diseases, these factors alone fail to fully account for the rapid rise in obesity and diabetes rates. The Environmental Obesogen Hypothesis posits that the burgeoning obesity epidemic is partially a consequence of the modulation of adipose development and function by synthetic chemicals (¹). Significant attention has focused on the capacity of environmental endocrine disrupting chemicals (EDCs) to promote adipogenesis (²), particularly through stimulation of the peroxisome proliferator activated receptor- γ (PPAR γ). Because PPAR γ is a principle regulator of adipocyte differentiation and function (³), compounds with the capacity to activate PPAR γ signaling are of great interest for understanding how synthetic chemicals might promote adipose accumulation. Indeed, several EDCs have been shown to act as PPAR γ agonists, including alkylated tin compounds, phthalates, flame retardants, and fungicides (reviewed in ref. (²)), suggesting that diverse exposures may alter adipose development and function.

The best studied of these environmental obesogens is tributyltin (TBT), which functions as a nanomolar agonist of both PPAR γ and retinoid X receptor- α (RXR α) (⁴). A number of studies have shown that TBT augments adipocyte differentiation in cell lines (^{1,5,6}), while also promoting fat deposition (⁷) and increased body weight (⁸) following *in vivo* exposure. Furthermore, *in utero* exposure to TBT increases adiposity postnatally (^{1,9}). Based on this strong data, studies of TBT form the foundation of the environmental obesogen hypothesis. Because of the metabolic benefits of smaller, more metabolically active adipocytes and the salutary metabolic effects of pharmacological PPAR γ agonists, e.g. thiazolidinediones (TZDs) (¹⁰), the pro-adipogenic effects of TBT would be predicted to improve energy homeostasis. However, in some experimental animal models, acute and chronic exposure to TBT resulted in metabolically deranged phenotypes (^{11,12}). This apparent paradox raises questions about the precise effects of TBT on adipose tissue development; therefore, studies were undertaken to delineate the contextual effects of TBT on adipocyte differentiation and to characterize the metabolic consequences of TBT-induced differentiation on mature adipocyte function.

Methods

3T3-L1 Culture and Differentiation

3T3-L1 preadipocytes were cultured in 10% calf serum as previously described (¹³). After reaching confluence, cells were refed for an additional two days at which point differentiation was initiated by the addition of Dulbecco's modified Eagle's medium

(DMEM; Mediatech, Manassas, VA) containing 10% fetal bovine serum (FBS; Aleken Biologicals, Nash, TX) and components of the differentiation cocktail: 167 nM insulin, a glucocorticoid receptor agonist (100 nM corticosterone (Cort) or 250 nM dexamethasone (Dex)), and/or 0.5 mM isobutylmethylxanthine (MIX) (all from Sigma, St. Louis, MO). After three days, cells were cultured for two additional days in DMEM plus 10% FBS and 167 nM insulin, after which assays were performed. The effects of TBT (5 or 50 nM) or the TZD troglitazone (Trog; 2.5 μ M) on 3T3-L1 differentiation were determined by incorporating TBT and/or Trog into the first 3 days of the differentiation protocol. All compounds were dissolved in 100% ethanol as vehicle (Sigma), with cells exposed to a final ethanol concentration of 0.1%. All co-exposure studies utilized TBT 50 nM and Trog 2.5 μ M.

Luciferase Assays

PPAR γ activity in undifferentiated 3T3-L1 preadipocytes was determined by luciferase assay as previously described (¹³). Briefly, subconfluent 3T3-L1 preadipocytes were transiently transfected with 2 μ g of luciferase construct containing two copies of the phosphoenolpyruvate carboxykinase PPAR γ response element into the pGL2-Promoter vector (Promega, Madison, WI) and 2 μ g of PPAR γ using Lipofectamine Plus (Invitrogen, Carlsbad, CA) over 16–18 h. Cells were then washed with PBS prior to 24 hour treatment with vehicle, TBT, or Trog in DMEM plus 10% calf serum. Cells were harvested and lysed, and luciferase activity determined as previously described (¹⁴).

Quantification of Adipocyte Lipid Accumulation

Lipid accumulation in differentiated 3T3-L1 adipocytes was determined by quantitative ORO staining. ORO (Sigma) was dissolved in isopropanol overnight at a concentration of 0.35% followed by 0.2 μ m filtration, dilution in water to a final concentration of 0.2%, and refiltration. Adipocytes were washed with PBS followed by 10% formalin fixation for 60 min. Cells were then washed with 60% isopropanol, allowed to dry, and stained with ORO for 10 min. Following multiple water washes, plates were dried at room temperature, ORO was eluted in 100% isopropanol, and 500 nm absorbance of the isopropanol solution measured using a Synergy H1 Hybrid Reader (BioTek, Winooski, VT).

Protein and Gene Expression Analyses

Preparation of whole-cell lysates, SDS-PAGE, and immunoblotting were performed as previously described (¹⁵). Western blots were probed with anti-adiponectin (Millipore, Billerica, MA), anti- β -actin, anti-CCAAT/enhancer binding protein- α (C/EBP- α), anti-perilipin, and anti-PPAR γ (Cell Signaling Technology, Beverly, MA) antibodies. Next, blots were probed with horseradish peroxidase-conjugated goat anti-mouse (adiponectin) or anti-rabbit (all other proteins) secondary antibodies (Bio-Rad, Hercules, CA). Relative protein expression was evaluated by densitometry using ImageJ version 1.47 (National Institutes of Health), with β -actin used to control for total protein recovery. RNA isolation, cDNA synthesis, and quantitative real time-PCR (qRT-PCR) were performed as previously described (¹⁶). Relative gene expression was evaluated by the Ct method (¹⁷), with β -actin used to control for total mRNA recovery. Primers were designed using Primer-BLAST

(National Center for Biotechnology Information) and obtained from Integrated DNA Technologies (Supplemental Table 1).

Glucose Uptake Assay

Following differentiation, glucose transport was assessed by the uptake of 2-deoxy-D-[³H]-glucose (³H-2-DG). Cells were incubated in assay media consisting of DMEM, 25 mM Hepes (pH 7.4), 0.5% FBS, and 5 mM glucose for 2.5 hours. Next, cells were washed with assay media lacking glucose, and incubated in this media in the presence or absence of 100 nM insulin for 30 min. The media were then spiked with 0.4 μ Ci ³H-2-DG in 200 μ M unlabeled 2-DG and incubated for 5 minutes. Probe uptake was terminated by placing the cells on ice and adding 200 mM unlabeled 2-DG, followed by washing with cold PBS. Cells were then scraped into ddH₂O, and cellular ³H-2-DG content was quantified by liquid scintillation counting.

Statistical Analyses

Between group differences were identified by repeated measures one-way analysis of variance (ANOVA) followed by Fisher's least significant difference *post hoc* test using GraphPad Prism version 6.0e (La Jolla, CA). All results represent N = 3 independent experiments, each performed in triplicate. $P < 0.05$ was considered significant for all analyses.

Results

TBT and Trog Stimulate PPAR γ Activity

To determine appropriate TBT and Trog concentrations for comparison, PPAR γ luciferase assays were performed. Relative to a vehicle-induced activity of 1.0, Trog 2.5 μ M increased luciferase expression to 1.8 ± 0.09 ($P < 0.001$); TBT 5 nM increased expression to 1.2 ± 0.12 ($P = 0.29$); TBT 50 nM increased expression to 1.6 ± 0.08 ($P < 0.01$); and TBT 100 nM increased expression to 1.6 ± 0.04 ($P < 0.01$). There was no statistically significant difference in luciferase activity between Trog 2.5 μ M and TBT 50 nM ($P = 0.20$); thus, these two treatments were considered comparable with regard to PPAR γ activation for the described studies, while also utilizing the lowest TBT concentration to achieve equivalent activation of PPAR γ signaling.

TBT and Trog Augment Lipid Accumulation

The well-characterized 3T3-L1 preadipocyte cell line is a model of adipogenesis with adipocyte differentiation induced using a cocktail that includes MIX to raise intracellular cAMP levels, the pharmacologic glucocorticoid Dex, and insulin (MDI). Adipocyte differentiation results in cellular lipid accumulation and expression of adipocyte-specific proteins. PPAR γ agonists induce or augment 3T3-L1 differentiation (¹⁸). To assess the relative capacity of TBT and Trog to promote adipocyte differentiation, these compounds were included in a submaximal differentiation cocktail, substituting the active, but less potent, murine glucocorticoid Cort for Dex (MCI). PPAR γ agonists were present only during the first 3 days of differentiation. Although TBT 5 nM did not increase lipid accumulation relative to the control ($P = 0.24$), TBT 50 nM and Trog comparably increased lipid

accumulation (Figure 1). Interestingly, the combination of Troglitazone and TBT 50 nM did not promote further lipid accumulation relative to TBT or Troglitazone alone.

TBT and Troglitazone Have Distinct Hormonal Requirements for Inducing Adipocyte-Specific Protein Expression

To characterize the conditions under which Troglitazone and TBT promote adipocyte-specific protein expression, adipocyte development was assessed in the presence of each individual component of the MCI cocktail in isolation. Adipocyte differentiation was determined 7 days post-induction by the expression of adipocyte-specific proteins, including perilipin (lipid droplet-associated protein), C/EBP α , PPAR γ (transcription factors), and adiponectin (adipokine). Expression of C/EBP α , an important early promoter of adipocyte differentiation⁽¹⁹⁾, provides a measure of relative adipogenesis within and across conditions at the time point investigated (Figures 2A (Vehicle), 2B (TBT), and 2C (Troglitazone)). In the presence of vehicle alone, neither Troglitazone nor TBT significantly increased expression of adipocyte-specific proteins, although there were trends toward increased expression with Troglitazone (Figure 2D). In the presence of MIX, however, TBT and Troglitazone both increased adipocyte-specific protein expression, although the effect of TBT was more pronounced; TBT increased expression of adiponectin, perilipin, and C/EBP α while Troglitazone only increased adiponectin expression (Figure 2E). In contrast, in the presence of Cort, Troglitazone upregulated expression of C/EBP α while TBT did not (Figure 2F). In the presence of insulin, both Troglitazone and TBT had similar effects on protein expression, increasing levels of perilipin and C/EBP α (Figure 2G). In addition, protein expression was interrogated following inclusion of Troglitazone or TBT into the full MDI cocktail to assess effects under maximally stimulatory conditions (Figure 2H). Troglitazone increased expression of PPAR γ , C/EBP α , and adiponectin relative to TBT. Additionally, while Troglitazone increased C/EBP α expression relative to vehicle, TBT co-exposure actually reduced adiponectin and perilipin expression relative to controls (Figure 2H).

TBT and Troglitazone Differentially Induce Adipocytic Gene and Protein Expression

Structurally distinct pharmacological PPAR γ agonists differentially regulate transcription of PPAR γ -responsive genes^(20,21). Because TBT lacks structural similarity to pharmacological PPAR γ agonists and because proper adipocyte function requires the expression of a multitude of PPAR γ -responsive genes, studies were undertaken to determine whether TBT and Troglitazone differentially altered expression of several PPAR γ -regulated adipocytic genes in the presence of a submaximal MCI differentiation cocktail. While both TBT and Troglitazone stimulated expression of some genes (e.g. PPAR γ , Figure 3A; *stearoyl-CoA desaturase-1 (SCD1)*, Figure 3B), the majority of genes investigated were responsive to Troglitazone but not TBT (e.g. *hexokinase 2 (HK2)*, *insulin receptor (IR)*, *oxidized low-density lipoprotein receptor-1 (OLR1)*, *adiponectin*, *C/EBP α* , and *glucose transporter-4 (Glut4)*, Figures 3C, 3D, 3E, 3F, 3G, and 3H, respectively). Importantly, while TBT co-treatment did not affect Troglitazone-stimulated gene expression for some genes (e.g. *HK2*, Figure 3C; *IR*, Figure 3D), TBT co-treatment attenuated or completely abolished Troglitazone-stimulated expression of several genes (e.g. PPAR γ , *SCD1*, *adiponectin*, *OLR1*, *C/EBP α* , and *Glut4*; Figures 3A, 3B, 3E, 3F, 3G, and 3H, respectively). Protein expression of the beneficial adipokine adiponectin was also assessed under MCI stimulation. Unlike Troglitazone, TBT did not increase expression relative to vehicle; furthermore, co-treatment with TBT markedly reduced Troglitazone-induced

adiponectin expression (Figure 3I). These results suggest that although TBT promotes adipocyte differentiation and lipid accumulation, the resultant gene and protein expression profile of adipocytes is distinct from the pharmacological PPAR γ agonist Trog; moreover, TBT alters Trog-induced expression in a gene-dependent manner.

Exposure to TBT during Differentiation Augments Basal Glucose Uptake

Adipose tissue is essential for metabolic homeostasis, storing energy in times of caloric surfeit and mobilizing energy in times of caloric scarcity. To assess how exposure to TBT and Trog during differentiation affect adipocyte metabolism, glucose uptake was interrogated in mature adipocytes under basal and insulin-stimulated conditions after exposure during the first three days of differentiation. Despite similar PPAR γ luciferase activity and lipid accumulation (Figure 1), TBT 50 nM increased basal glucose uptake, whereas Trog exerted no effect; co-exposure maintained the TBT effect (Figure 4A). Under insulin-stimulated conditions, exposure to Trog, but not TBT, augmented insulin-stimulated glucose uptake; the effect of Trog was maintained with co-exposure (Figure 4B). At the concentrations tested, these findings suggest that TBT and Trog differentially alter adipocyte glucose handling despite similar activation of PPAR γ signaling and lipid accumulation.

Discussion

TBT is an organotin that has historically been used as a booster biocide in marine paints, a fungicide on fruit crops and in wood preservatives, a disinfectant in textiles, and a polyvinylchloride (PVC) resin stabilizer (22). Human exposure principally occurs through contaminated water and seafood (23) as well as PVC-containing devices (24). While its use as a booster biocide has been curtailed by international treaties (25), its environmental persistence and continued use in developing countries render it an ongoing human health threat. A variety of studies have shown that TBT has the capacity to induce adipocyte differentiation (1,5,6); however, the effects of this chemical on adipocyte development and physiology are more complex and nuanced than initially appreciated and do not simply recapitulate the action of other PPAR γ agonists.

In the present study, both TBT and Trog increased adipocyte differentiation (Figures 1 and 2); however, TBT exposure generated a unique gene expression profile relative to Trog (Figures 3A–H). Moreover, these studies show for the first time that the co-stimulatory conditions under which TBT promotes adipogenesis differ from those under which a TZD increases adipocyte development (Figure 2). Most notably, TBT increased expression of three adipocyte-specific proteins in the presence of elevated levels of cAMP brought about by the phosphodiesterase inhibitor MIX (Figure 2E). Increases in intracellular cAMP stimulate adipogenic differentiation by activating cAMP-responsive element-binding protein (CREB) (26), which induces a temporally regulated transcriptional cascade, increasing levels of multiple transcription factors, including C/EBP β , C/EBP α , and PPAR γ (19). Additionally, intracellular increases in cAMP may increase production of endogenous PPAR γ ligands (27). Importantly, unlike Trog, TBT exhibits dual agonism on both RXR α and PPAR γ (4). Because RXR α is a permissive partner of PPAR γ , activation of RXR α independently increases PPAR γ transcriptional activity (28). Indeed, in a murine bone

marrow stem cell line, the RXR α agonist bexarotene promoted adipocyte differentiation (29). In models of acute myeloid leukemia, RXR agonist-induced cellular differentiation was contingent upon coordinate stimulation of cAMP signaling (30,31). Similar crosstalk between cAMP signaling and RXR α may explain the potentiation of TBT-induced adipogenesis with co-treatment with MIX. Importantly, increased sympathetic tone increases cAMP production through adrenergic signaling (32), suggesting that pathophysiologic states characterized by increased adrenergic tone, e.g. obstructive sleep apnea (32,33), may augment TBT-induced adipocyte development. Finally, because RXR signaling appears to be more important for human adipogenesis compared to mice (6), TBT agonism of RXR α in promoting human adipogenesis may be understated in murine studies.

Adipose tissue plays a central role in metabolic homeostasis through the regulated storage and mobilization of energy stores and the secretion of metabolic hormones (i.e. adipokines). Endogenous hormones modulate shifts in metabolic states, with insulin primarily stimulating the transition to nutrient uptake and storage (34). In the present study, TBT increased adipogenesis in the presence of insulin alone (Figure 2G), suggesting that TBT may be an especially potent adipogenic signal under hyperinsulinemic conditions, such as the postprandial state or in type 2 diabetes. Additionally, exposure to TBT during differentiation generated mature adipocytes with increased capacity for basal glucose uptake, an effect not seen with Trog (Figure 4A). If recapitulated *in vivo*, this increase in non-insulin-mediated glucose uptake could promote continuous expansion of adipose fat stores even under fasting conditions.

Adipose also modulates global energy homeostasis through the secretion of adipokines such as adiponectin. Among its many physiological effects, adiponectin increases global insulin sensitivity (35) and promotes β -cell survival (36). The present studies demonstrate that TBT, in contrast to Trog, decreases adiponectin protein expression when differentiation is maximally stimulated (Figure 2H), suggesting that TBT has the capacity to modulate adiponectin production in a manner expected to promote metabolic dysfunction. This suggests that the insulin resistance (12) and β -cell apoptosis (37) observed following *in vivo* TBT exposure could arise, at least partially, as a consequence of direct TBT disruption of adipose adiponectin production. Additionally, co-treatment with TBT completely abolished Trog-induced adiponectin gene expression (Figure 3F) and drastically blunted Trog-induced adiponectin protein expression (Figure 3I). In human studies, Trog increased circulating adiponectin levels, and concentrations of this beneficial adipokine both before and after treatment correlated with glucose disposal rates, suggesting that adiponectin may be an important mediator of the salutary effects of Trog on systemic glucose metabolism (38). Thus, it is possible that exposure to TBT may antagonize the anti-diabetic efficacy of Trog, and perhaps other TZDs. Finally, several EDCs have been shown to reduce adiponectin expression, including polychlorinated biphenyl-77 (39), bisphenol A (40), and tolylfluanid (16), suggesting that adiponectin may be a common target of metabolically disruptive EDCs.

Although the present studies have identified several novel aspects of TBT-induced adipogenesis, there are several limitations. First, these studies were conducted in the 3T3-L1 cell line, which models preadipocyte-to-adipocyte differentiation; potential effects on earlier stages of differentiation were not explored, e.g. mesenchymal stem cell commitment to the

adipocyte lineage. Second, TBT and Trog exposure were restricted to the first three days of the differentiation protocol during which the cells undergo tremendous changes in gene expression. Whether the effects of TBT would be modulated by continued exposure throughout differentiation requires further investigation. Third, this investigation focused on a single time point for assessing markers of adipocyte differentiation and effects on glucose uptake. As such, it is possible that alternative exposure paradigms, modeling other concentrations and durations of treatment, may yield further insights into the differential effects of TBT and Trog in modulating adipocyte development and physiology. Fourth, the present studies employ an *in vitro* model to replicate an *in vivo* phenomenon. *In vivo* exposure is complicated by the interplay of multiple metabolic tissues in the regulation of energy homeostasis; however, specific interrogation of the functional state of adipose tissue after *in vivo* TBT exposure is warranted given the present results, as is more comprehensive metabolic profiling of TBT-exposed mice. Finally, Trog was utilized as the model pharmacological PPAR γ agonist; however, given the ligand-specificity of PPAR γ activity (20,21), interrogation of other pharmacological TZDs and EDC-agonists of PPAR γ may further illuminate the unique adipocytic disruptions resulting from TBT exposure.

These novel findings suggest that all adipocytes generated by activation of PPAR γ signaling are not created equal. Furthermore, these studies suggest that differences in the resulting adipocyte may have profound effects on the function of EDC-induced adipose tissue, and consequentially, global energy homeostasis. This adipocyte heterogeneity supports a more expansive approach to characterizing environmental obesogens, with directed interrogation of the myriad pathways by which adipocytes regulate metabolism. Such analyses will greatly expand our understanding of the marked adipose heterogeneity that results from exposure to environmental toxicants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Study Importance

- Prior studies have shown that tributyltin (TBT) is a dual agonist of the peroxisome proliferator activated receptor- γ (PPAR γ) and the retinoid X receptor- α , with the capacity to augment adipocyte differentiation *ex vivo* and adipose accumulation *in vivo*.
- The present study delineates the adipogenic conditions under which TBT promotes preadipocyte-to-adipocyte differentiation and demonstrates novel differences between adipocytes exposed during differentiation to TBT or the pharmacological PPAR γ agonist troglitazone, including differential gene and protein expression as well as alterations in glucose uptake.
- Tributyltin exposure during differentiation, in contrast to troglitazone, produces an adipocyte with metabolically deleterious characteristics, including reduced expression of the insulin-sensitizing adipokine adiponectin.

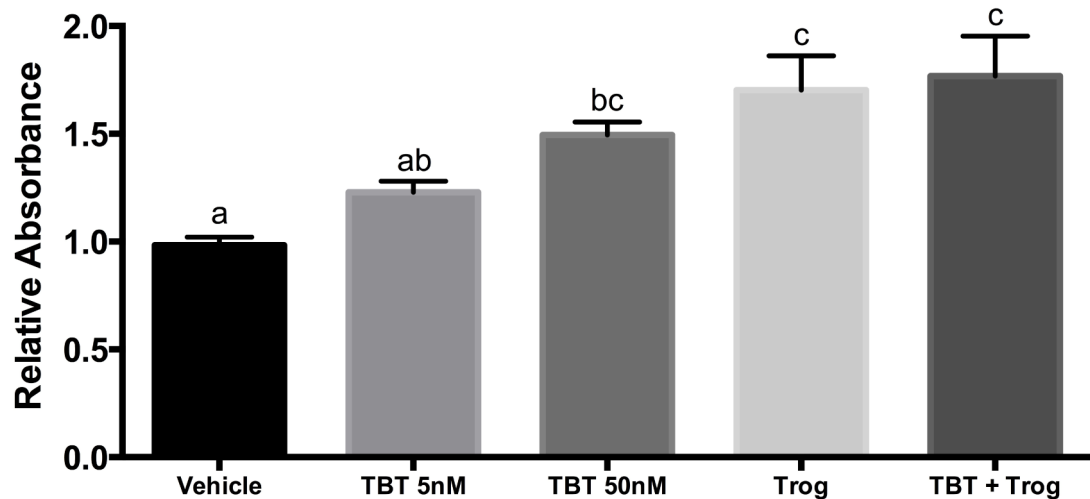


Figure 1. Exposure to the environmental and pharmacological PPAR γ agonists TBT and Trog during adipocyte differentiation promotes adipocyte lipid accumulation

Two days after reaching confluency, 3T3-L1 preadipocytes treated with a submaximal MCI differentiation cocktail: 10% FBS in DMEM containing 167 nM insulin, 0.5 mM isobutylmethylxanthine, and 100 nM corticosterone in the presence of either vehicle, TBT (5 or 50 nM), 2.5 μ M Trog, or 50 nM TBT plus 2.5 μ M Trog. Following three days in differentiation media, media was replenished with 10% FBS in DMEM containing 167 nM insulin. Following two additional days in insulin-containing media, cells were assessed for lipid accumulation by ORO staining, with absorbance measured at 500 nM and increased absorbance indicative of increased lipid accumulation. Three independent experiments were performed in triplicate and normalized to a null condition. Differences were determined by ANOVA, and treatments not sharing a letter were significantly different with $P < 0.05$. Data are presented as means \pm S.E.M. TBT, tributyltin; Trog, troglitazone.

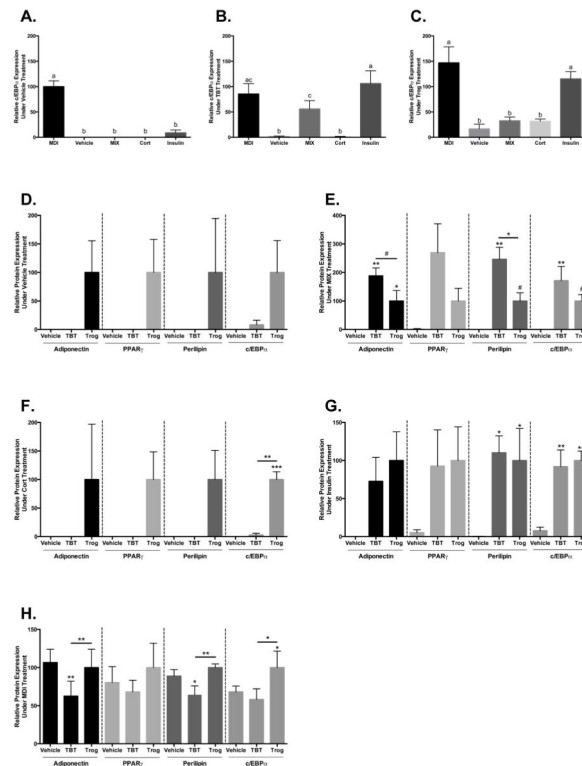


Figure 2. TBT and Trog promote adipocyte differentiation in a context-dependent manner

Two days after reaching confluency, 3T3-L1 preadipocytes were treated with differentiation media: 10% FBS in DMEM containing either the full MDI differentiation cocktail (0.5 mM MIX, 250 nM dexamethasone, and 167 nM insulin) or individual components of the submaximal MCI differentiation cocktail (0.5 mM MIX, 100 nM Cort, or 167 nM insulin) in the presence or absence of 50 nM TBT or 2.5 μM Trog as indicated. Following three days in differentiation media, media was replenished with 10% FBS in DMEM containing 167 nM insulin. Following two additional days in insulin-containing media, whole cell lysates were collected, processed, and resolved by immunoblotting. Relative protein expression of C/EBPα across conditions provides a measure of adipogenesis within vehicle (Panel A), TBT (Panel B), and Trog (Panel C) treatment groups. Expression of adiponectin, PPARγ, perilipin, and C/EBPα were assessed in vehicle (D), MIX (E), Cort (F), insulin (G), and MDI conditions (H). Relative protein expression was determined by densitometry and normalized to β-actin to control for total protein recovery, with three independent experiments performed in triplicate. Differences in protein expression were determined by ANOVA; data are presented as means ± S.E.M. Panels A–C were normalized to the mean of the MDI-vehicle treatment condition, and conditions not sharing a letter were significantly different at $P < 0.05$. Panels D–H were normalized to the mean of Trog treatment for each protein investigated. # $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. C/EBPα, CCAAT/enhancer binding protein-α; Cort, corticosterone; MIX, isobutylmethylxanthine; PPARγ, peroxisome proliferator activated receptor-γ; TBT, tributyltin; Trog, troglitazone.

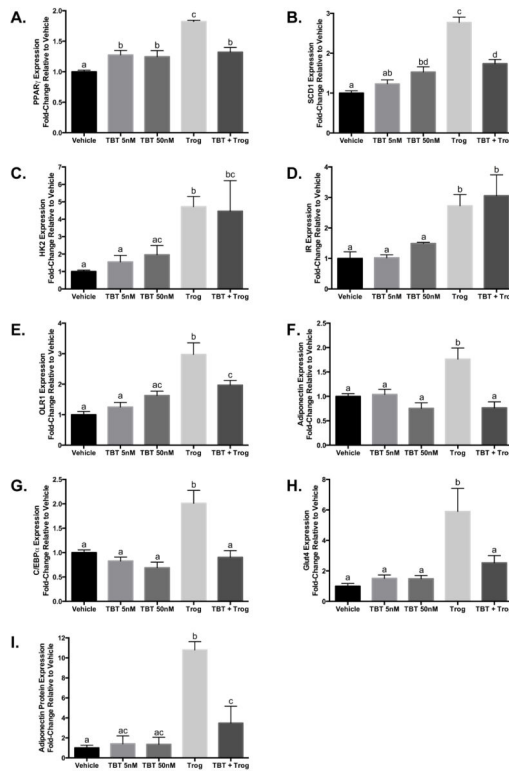


Figure 3. TBT and Trog exposure during differentiation promote distinct gene and protein expression profiles in mature adipocytes

Two days after reaching confluency, 3T3-L1 preadipocytes were provided submaximal MCI differentiation media: 10% FBS in DMEM containing 167 nM insulin, 0.5 mM MIX, and 100 nM corticosterone in the presence or absence of TBT (5 or 50 nM), 2.5 μM Trog, or 50 nM TBT plus 2.5 μM Trog. Following three days in differentiation media, media was replenished with 10% FBS in DMEM containing 167 nM insulin. Following two additional days in insulin-containing media, either RNA or whole cell lysates were collected. Gene expression of PPAR γ (Panel A), SCD1 (Panel B), HK2 (Panel C), IR (Panel D), OLR1 (Panel E), adiponectin (Panel F), C/EBP α (Panel G), and Glut4 (Panel H), were assessed by qRT-PCR, and normalized to β -actin to control for RNA recovery. Whole cell lysates were processed and resolved by immunoblotting, with relative protein expression of adiponectin assessed by densitometry and normalized to β -actin to control for total protein recovery (I). N=3–10 performed in triplicate and normalized to a vehicle average of 1. Differences in gene and protein expression were determined by ANOVA; treatments not sharing a letter were significantly different at $P < 0.05$. Data are presented as means \pm S.E.M. C/EBP α , CCAAT/enhancer binding protein- α ; Glut1, glucose transporter 1; Glut4, glucose transporter 4; HK2, hexokinase 2; IR, insulin receptor; OLR1, oxidized low-density lipoprotein receptor 1; PPAR γ , peroxisome proliferator activated receptor- γ ; SCD1, stearoyl-CoA desaturase-1; TBT, tributyltin; Trog, troglitazone.

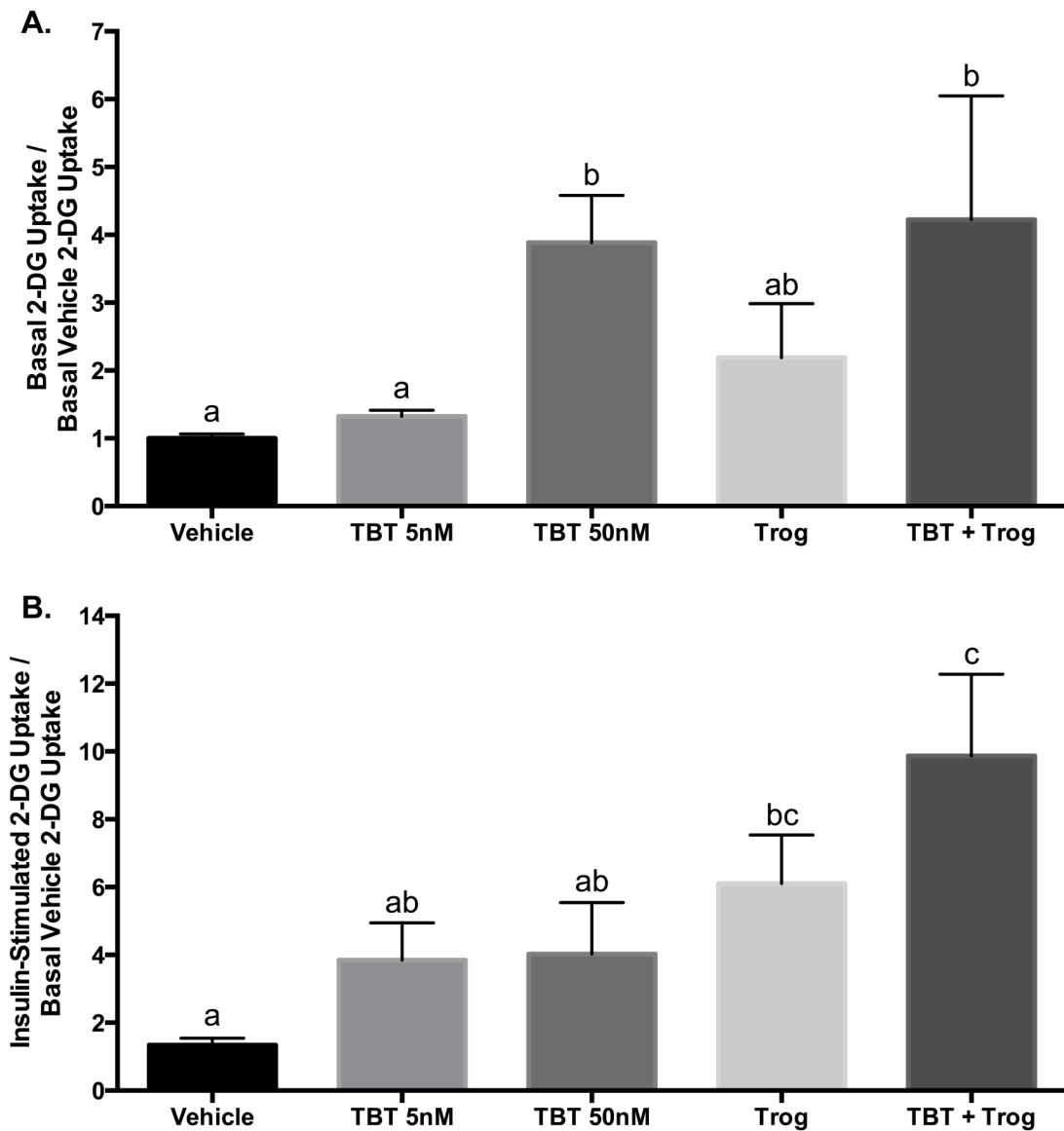


Figure 4. TBT exposure during differentiation augments basal glucose uptake in mature adipocytes

Two days after reaching confluency, 3T3-L1 preadipocytes were provided submaximal MCI differentiation media: 10% FBS in DMEM containing 167 nM insulin, 0.5 mM MIX, and 100 nM corticosterone in the presence or absence of TBT (5 or 50 nM), 2.5 μ M Trog, or 50 nM TBT plus 2.5 μ M Trog. Following three days in differentiation media, media was replenished with 10% FBS in DMEM containing 167 nM insulin. Following two additional days in insulin media, glucose uptake was assessed under basal (Panel A) or insulin-stimulated (100 nM) conditions (Panel B) by supplementing media with ^3H -2-DG for 5 minutes and glucose uptake determined by liquid scintillation counting, with four independent experiments performed in triplicate and normalized to basal uptake under vehicle treatment. Differences in glucose uptake were determined by ANOVA; treatments

not sharing a letter were significantly different at $P < 0.05$. Data are presented as means \pm S.E.M. 2-DG, 2-deoxy-D-glucose; TBT, tributyltin; Trog, troglitazone.

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