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J Kim

Q Sun

Y Yue

Kyong-Sup Yoon
Southern Illinois University Edwardsville, kyoon@siue.edu

K -Y Whang

See next page for additional authors

Authors
J Kim, Q Sun, Y Yue, Kyong-Sup Yoon, K -Y Whang, J M. Clark, and Y Park

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4,4'-Dichlorodiphenyltrichloroethane (DDT) and 4,4'-dichlorodiphenyldichloroethylene (DDE) promote adipogenesis in 3TL1 adipocyte cell culture

Jonggun Kim[a,1], Quancai Sun[b,1], Yiren Yue[b], Kyong Sup Yoon[c], Kwang-Youn Whang[a], J. Marshall Clark[d], and Yeonhwa Park[b,*]

[a] Division of Biotechnology, Korea University, Seoul 136-713, Republic of Korea,

[b]Department of Food Science, University of Massachusetts, Amherst, 102 Holdsworth Way, Amherst, MA 01003, United States,

[c]Department of Biological Sciences and Environmental Sciences Program, Southern Illinois University, Edwardsville, IL 62026, United States,

and [d]Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, 637 North Pleasant Street, Amherst, MA 01003, United States

[1] J. Kim and Q. Sun contributed equally.

[*]To whom correspondence should be addressed

Department of Food Science, University of Massachusetts

102 Holdsworth Way, Amherst, MA 01003

Telephone: (413) 545-1018, Fax: (413) 545-1262; e-mail: ypark@foodsci.umass.edu

Running title: DDT and DDE potentiate lipid accumulation

Abbreviations: AKT, protein kinase B; AMPKα, AMP-activated protein kinase-α; ACC, acetyl-CoA carboxylase; ATGL, adipose triglyceride lipase; CEBPα, CCAAT/enhancer-binding protein α; FAS, fatty acid synthase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLUT 4, glucose transporter 4; HSL, Hormone sensitive lipase; LPL, lipoprotein lipase; PPARγ, peroxisome-proliferator activated receptor-γ; TG, triacylglyceride.

Abstract

4,4'-Dichlorodiphenyltrichloroethane (DDT), a chlorinated hydrocarbon insecticide, was extensively used in the 1940s and 1950s. DDT is mainly metabolically converted into 4,4'-dichlorodiphenyldichloroethylene (DDE). Even though most countries banned DDT in the 1970s, due to the highly lipophilic nature and very stable characteristics, DDT and its metabolites are present ubiquitously in the environment, including food. Recently, there are publications on relationships between exposure to insecticides, including DDT and DDE, and weight gain and altered glucose homeostasis. However, there are limited reports regarding DDT or DDE and adipogenesis, thus we investigated effects of DDT and DDE on adipogenesis using 3T3-L1 pre-adipocytes. Treatment of DDT or DDE resulted in increased lipid accumulation accompanied by increased expression of CCAAT/enhancer-binding protein α (C/EBPα), peroxisome-proliferator activated receptor-γ (PPARγ), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), adipose triglyceride lipase, and leptin. Moreover, treatment of DDT or DDE increased protein levels of C/EBPα, PPARγ, AMP-activated protein kinase-α (AMPKα), and ACC, while significant decrease of phosphorylated forms of AMPKα and ACC were observed. These finding suggest that increased lipid accumulation caused by DDT and DDE may mediate AMPKα pathway in 3T3-L1 adipocytes.

Keywords: DDT, DDE, 3T3-L1, adipogenesis, adipocyte differentiation, lipogenesis

1. Introduction

4,4'-Dichlorodiphenyltrichloroethane (DDT), the most important chlorinated hydrocarbon insecticide ever marketed, was widely and extensively used in the 1940s and 1950s to control insects for protection of both agriculture and human health [1, 2]. In fact, DDT is still used in malaria-endemic areas (sub-Saharan Africa and India) to control malaria vectors, although most countries banned the use of DDT in the 1970s [3]. Its insecticidal properties were first discovered by Paul Müller in 1939, and it acts on the nervous system through modulation of voltage-sensitive sodium channels (VSSCs) described later by Narahashi and Yamasaki [1, 2, 4]. This modulation is known to cause the characteristic symptom called "DDT jitters", which can be best described as the repetitive discharge in the nerve that leads to whole body tremors as a result of increased "depolarizing after-potentials" [2, 4, 5]. It is known that DDT can be metabolically converted into 4,4'-dichlorodiphenyldichloroethane (DDD) and 4,4'-dichlorodiphenyldichloroethylene (DDE) by reductive dechlorination and dehydrochlorination, where the latter is the more common metabolite of DDT [6]. Evidence of DDT and its metabolites and eggshell thinning in certain bird populations drew significant awareness of these compounds [7]. It has been suggested that this is due to the inhibition of prostaglandin synthase, however, others suggested multiple mechanisms contributing to eggshell thinning by DDT and its metabolites [8-11].

The indiscriminate use of DDT in earlier times along with the highly lipophilic nature and extremely slow degradation of this compound and its metabolites *in vivo* and *in vitro* as well as in the environment have resulted in the ubiquitous distribution of these compounds in the environment including food, especially in fatty foods [2, 12, 13]. Along with other environmental contaminants, DDT and DDE are currently defined as endocrine disruptors [14-

18]. Moreover, a growing body of literature shows relationships between exposure to insecticides, including DDT and DDE, and adverse health effect in humans such as weight gain and altered glucose homeostasis [19-34]. In particular, Lee et al. [30] reported the serum levels of persistent organic pollutants including DDE positively correlated with body mass index (BMI). Currently there are two publications investigating the role of DDT or DDE on adipogenesis using either 3T3-L1 or 3T3-F442A adipocytes [35, 36]. Moreno-Aliaga and Matsumura [36] reported that DDT promoted adipocyte differentiation by modification of key transcription factors of this process, such as CCAAT/enhancer-binding protein β (C/EBPβ), peroxisome-proliferator activated receptor-γ (PPARγ), and C/EBPα. Others reported the role of DDE and other organochlorine insecticides, oxychlordane and dieldrin, on adipogenesis, fatty acid uptake and adipokine productions in 3T3-L1 adipocytes. Based on these findings, along with our previous reports that other types of insecticides promoted adipogenesis in 3T3-L1 adipocytes [37-39], we determined the role of DDT and DDE in adipogenesis in this model.

2. Materials and Methods

2.1. Materials

3T3-L1 pre-adipocytes were purchased from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine serum (BS), methylisobutylxanthin, dexamethasone, insulin, dimethyl sulfoxide, DDT, DDE, and protease inhibitor cocktail were purchased from Sigma-Aldrich Co. (St. Louis, MO). The amounts of triglyceride (TG) and protein were quantified using kits from Genzyme Co. (Cambridge, MA) and Bio-Rad Co. (Hercules, CA), respectively. Radioimmunoprecipitation assay (RIPA) buffer with EDTA and EGTA was purchased from Boston Bioproducts Inc.

(Ashland, MA). Primary rabbit antibodies for PPARγ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Primary rabbit antibodies for acetyl-CoA carboxylase (ACC), phosphorylated ACC (pACC), AMP-activated protein kinase-α (AMPKα), phosphorylated AMPKα (pAMPKα), and CCAAT/enhancer-binding protein α (C/EBPα) and horseradish peroxidase conjugated goat anti-rabbit IgG (the secondary antibody) were purchased from Cell Signaling Technology (Berberly, MA).

2.2. Cell culture

The preadipose cell line 3T3-L1 was originally developed by clonal expansion from murine Swiss 3T3 cells, and has been widely used as an *in vitro* model for adipogenesis and the biochemistry of adipocytes [40]. 3T3-L1 pre-adipocytes were cultured as previously described with minor modifications [41]. Briefly, 3T3-L1 pre-adipocytes were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine serum (BS) to confluence. At two days after confluence (designated as day 0), adipocyte differentiation was induced with a mixture of methylisobutylxanthin (0.5 mM), dexamethasone (1 μM), and insulin (1 μg/ml) in DMEM containing 10 % FBS. On day 2, this medium was replaced with DMEM containing 10 % FBS and insulin (1 μg/ml). On day 4 and thereafter, medium consisting of DMEM plus 10 % FBS was subsequently replaced with fresh medium at 2 day intervals for 4 days.

Cells were treated with either DDT or DDE at final concentrations of 10 or 20 μM by adding stock solution of 100 mM in dimethyl sulfoxide. DDT or DDE treatment of 3T3-L1 cells started at day 0 and cells were harvested at day 8. Control was treated with dimethyl sulfoxide only and all treatments had dimethyl sulfoxide at a final concentration of 0.02%. These

concentrations of DDT/DDE had no effects on cell viability as measured by a 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) based assay (data not shown) [42].

2.3. Triglyceride quantification

After 8 days of adipogenic differentiation, cells were washed twice with phosphate-buffered saline (PBS) and harvested by scraping in PBS containing 1% Triton-X. Cells were sonicated to obtain homogenous samples. After centrifugation at 500×g for 5 min at 4°C, the amount of triglyceride (TG) in the supernatant was measured using a colorimetric assay (Triglyceride-SL assay kit; Genzyme Diagnostics, Charlottetown, PE, Canada). Protein concentrations were measured by using the Bio-Rad protein DC assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard, and TG content was normalized with protein concentration.

2.4. mRNA expression analysis

Total RNA was extracted from cells using Trizol® reagent under RNase free conditions. Total RNA was reverse-transcribed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). mRNA expression levels of CCAAT/enhancer-binding protein α (CEBPα), PPARγ, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), and leptin were analyzed from 3T3-L1 adipocytes. Real-time PCR was performed using a StepOne Plus real time PCR machine (Applied Biosystems, Carlsbad, CA) and Taqman® probe-based gene expression analysis (Applied Biosystems, Carlsbad, CA). Respective integrated sequences for Taqman® gene expression were NM_007678.3 (C/EBPα), NM_001127330.2 (PPAR-γ), NM_133360.2 (ACC),

NM_007988.3 (FAS), NM_001039507.1 (HSL), NM_008509.2 (LPL), NM_001163689 (ATGL), NM_009204.2 (GLUT 4), and NM_008493.3 (leptin), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_008084.2) as an internal standard. Threshold values were analyzed using comparative CT (ΔΔCT) method [43].

2.5. Immunoblotting

After treatment, cells were lysed using RIPA buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Protein concentrations were determined using the protein DC assay kit (Bio-Rad Co., Hercules, CA) with bovine serum albumin standard. Aliquots from the cell lysates containing 37 μg of protein were separated using 8% or 10% SDS-polyacrylamide gel and transferred to an Immobilin P membrane (Millipore, Bedford, MA). Primary antibodies from rabbits were diluted as recommended by producers. GAPDH was used as an internal control to normalize protein content. Horseradish peroxidase conjugated goat anti-rabbit IgG was used as the secondary antibody. Detection was performed using a Clarity™ Western ECL Substrate Kit (Bio-Rad Co., Hercules, CA) with an Image Station 4000MM (Carestream Health, New Haven, CT) and image and results were quantified using Image J software.

2.6. Statistical analyses

Data were analyzed by the analysis of variance procedure (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC). Significant differences between treatments were determined using Tukey's multiple-range test. Significance of differences was defined at the $P<0.05$ level.

3. Results

Figure 1 shows the effects of DDT and DDE on triglyceride accumulation in 3T3 adipocytes. When cells were treated with either of these compounds there was significant increase in TG accumulation at 10 μM of DDT and both 10 and 20 μM of DDE compared to control in this model.

Figure 2 shows influences of DDT and DDE on genes involved in regulation of adipocyte differentiation and lipid metabolism. Treatment with DDT and DDE consistently increased expression of key genes involved in adipocyte differentiation, C/EBPα and PPARγ, compared to control, greater effect on PPARγ than C/EBPα (Fig. 2A & 2B). In addition, both DDT and DDE increased expression of FAS and ACC, key enzymes for lipogenesis (Fig. 2C & 2D). Expression of ATGL, one of the important genes of adipocyte differentiation regulated by PPARγ and/or lipolysis [44], was also increased after DDT and DDE treatments compared to control (Fig. 2E). HSL, gene regulate lipolysis, was also increased by both DDT and DDE (Fig. 2F). Only significant effects of 20 μM of DDE was observed in LPL compared to control (Fig. 2G). Expression of leptin was greatly increased by DDT and DDE treatments, 5-14 fold, over control (Fig. 2H). There was no significant difference in GLUT 4 expression by these insecticides (Fig. 2I).

We further analyzed effects of DDT and DDE on adipogenesis by measuring protein expressions of key regulators of adipogenesis (Figure 3). Treatment of DDT and DDE increased protein expression of C/EBPα compared to control, although it did not reach significance with and PPARγ (Fig. 3A & 3B). AMP-activated protein kinase-α (AMPKα) is a master regulator of energy production and lipid metabolism in the cell [45, 46]. When 3T3-L1 cells were treated with DDT and DDE, significant decrease of phosphorylated form of AMPKα were observed

without affecting total AMPKα (Fig. 3C & 3D), resulting in significant reduction of this ratio (Fig. 3E). These suggest reduced activation of AMPKα by DDT and DDE. Expression of ACC (active form), a downstream target of AMPKα, was significantly increased, while phosphorylation of ACC (inactive form) was significantly reduced by DDT and DDE treatment (Fig. 3F, 3G & 3H). This would result in increased lipogenesis by DDT and DDE compared to control. These finding suggest that DDT and DDE elicit effects on adipogenesis via post-translational regulation of AMPKα, contributing to altered adipocyte differentiation and lipid accumulation in this model.

4. Discussion

Results from this report suggest that exposure to DDT and DDE contribute to increased lipid accumulation via altered adipogenesis in 3T3-L1 adipocyte model. This observation is similar to that seen by others that DDT and DDE influence adipocyte differentiation and lipid metabolism [35, 36]. However, this is the first to compare the effects of DDT and DDE in the same study, where we observed greater effects of DDE on adipogenesis compared to DDT. We further expanded our understanding that both DDT and DDE influence adipogenesis via post-translational modulation of AMPKα phosphorylation.

Previously, Moreno-Aliaga and Matsumura [35] reported that treatment with DDT in 3T3-L1 and 3T3-F442A adipocytes resulted in increased lipid accumulation and adipocyte differentiation by modulating protein levels of CCAAT/enhancer-binding protein β (C/EBPβ), PPARγ, and C/EBPα. Consistent with these results we observed this not only with DDT but also with DDE treatment on PPARγ and C/EBPα in the current report. Howell and Mangum [36] reported that DDE potentiated adipogenesis, fatty acid uptake, and adipokine productions in 3T3-

L1 adipocytes, while no difference in lipid accumulation was observed compared to control. Our current results clearly showed that both DDT and DDE potentiated lipid accumulation, while inducing adipogenesis and increasing leptin expression (one of major adipokines). The discrepancy may be due to the differences in methods used to quantify the total lipid.

It has been reported that serum levels of DDT and DDE ranged from 0.05-0.08 nM and 7-13 nM, respectively, or 32-2,542 ng DDE/g lipids in human serum [22, 47, 48]. Compared to serum levels of DDT or DDE found in the human, the concentration we have used in this study is very high. However, DDE has been detected at levels as high as 2,542 ng/g lipids in serum with an average of 500 ng/g lipids, estimated to be equivalent to 1.4-7.3 μM [47]. In addition, these organochlorine insecticides may reach high concentrations in certain compartments of cells as they are known to bio-accumulate in human tissues, particularly in adipocytes [49]. Thus, the concentrations we have tested may have biological relevance.

In the current study, we did not measure the effect of DDT or DDE on binding specificity of potential regulators of adipogenesis. However, previously it was reported that other organochlorines and other types of insecticides all potentiate adipogenesis [36, 38, 39]. It is known that DDE is a slightly more water-soluble metabolite of DDT, although both compounds are extremely lipophilic [6]. Thus, we speculate any difference between the effects of DDT and DDE on adipogenesis may originate from availability in cell compartment, rather than binding specificity on particular target genes as previously described [50].

Howell and Mangum [36] further reported that organochlorine insecticides tested (DDE, dieldrin, and oxychlordane) showed no effects on insulin stimulated fatty acid uptake but increased basal fatty acid uptake. We have not tested effects of these chemicals on insulin-mediated responses in the current study. However, we recently reported the potential role of two

other types of insecticides on adipogenesis in 3T3-L1 adipocytes and impaired insulin stimulated glucose uptake in C2C12 myotubes [37-39]. We tested imidacloprid (a neonicotinoid insecticide) and permethrin (a pyrethroid insecticide), both of which potentiated TG accumulation via similar mechanisms that we observed with DDT and DDE in this report. Particularly, insecticidal activities of permethrin are similar to that of DDT and DDE, although imidacloprid is known to act on nicotinic acetylcholine receptors (nAChRs) [51, 52]. These findings suggest that DDT and DDE may possibly influence insulin-mediated responses similar to imidacloprid and permethrin as well as a common biochemical mechanism underlying these insecticides with regards to adipogenesis. This also implies that other insecticides that we have not tested may result in similar responses in adipogenesis.

It is important to note that imidacloprid and permethrin did not influence expression of leptin levels, while DDT and DDE significantly increased leptin expression in the current study. This implies that insecticides may share similar mechanisms in adipogenesis while additional mechanism(s) by these insecticides may exist. Similarly, different mechanisms were previously observed in insulin-stimulated glucose uptake for imidacloprid and permethrin previously [37, 38]. Thus, further studies using *in vivo* models as well as the role of DDT and DDE in glucose uptake response to insulin will be needed to understand its role in comparison to other types of insecticides with regards to human health perspective, particularly for type 2 diabetes.

In the current study, we observed increased expression of ATGL, which represents increased adipocyte differentiation and/or lipolysis [44]. And our results on HSL suggest that both DDT and DDE potentiated lipolysis. Compared to other two insecticides tested previously, the increase of lipid accumulation caused by DDT and DDE in the current study is relatively low, about 20% increase over control compared to about 100% increase by imidacloprid or

permethrin [37, 38]. This may have been due to increased lipolysis by DDT and DDE. However, Howell and Mangum [36] reported that DDE did not induce lipolysis. Thus, it will be likely that increased ATGL by DDT and DDE in the current study may represent in part increased adipocyte differentiation in addition to increased lipolysis.

Our current study concludes that the roles of DDT and DDE in adipogenesis are via altered adipocyte differentiation and lipid metabolism. Along with the current effort to understand obesity epidemics, these results may help identify fundamental underlying links between insecticide exposures and obesity and its co-morbidities. However, additional future *in vivo* studies as well as epidemiological studies are necessary to further elucidate the significance of the current study.

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Figure 1. Treatment of DDT and DDE increased triacyglyceride accumulation in 3T3-L1 adipocytes. Cells were treated with DDT or DDE for 8 days. Numbers are mean±S.E. (n=4). Means with different letters are significantly different at $P<0.05$.

Figure 2. Effects of DDT and DDE on gene expression of molecular mediators of adipogenesis. C/EBP-α, CAATT element binding protein-α; PPAR-γ, peroxisome proliferator-activated receptor-γ; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; GLUT 4, glucose transporter 4. Cells were treated with permethrin for 8 days. GAPDH was used as an internal control. Numbers are mean±S.E. (n=4). Means with different letters are significantly different at $P<0.05$.

Figure 3. Effects of DDT and DDE on protein levels of molecular mediators of adipogenesis. C/EBP-α, CAATT element binding protein-α; PPAR-γ, peroxisome proliferator-activated receptor-γ; AMPKα, AMP-activated protein kinase-α (inactive form); pAMPKα, phosphorylated AMPKα (active form), ACC, acetyl-CoA carboxylase (active form); pACC, phosphorylated ACC (inactive form). Cells were treated with permethrin for 8 days. GAPDH was used as an internal control. Numbers are mean±S.E. (n=4). Means with different letters are significantly different at $P<0.05$.

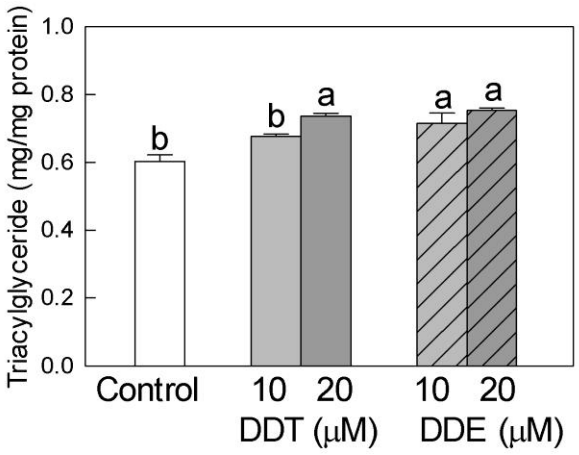


Figure 1

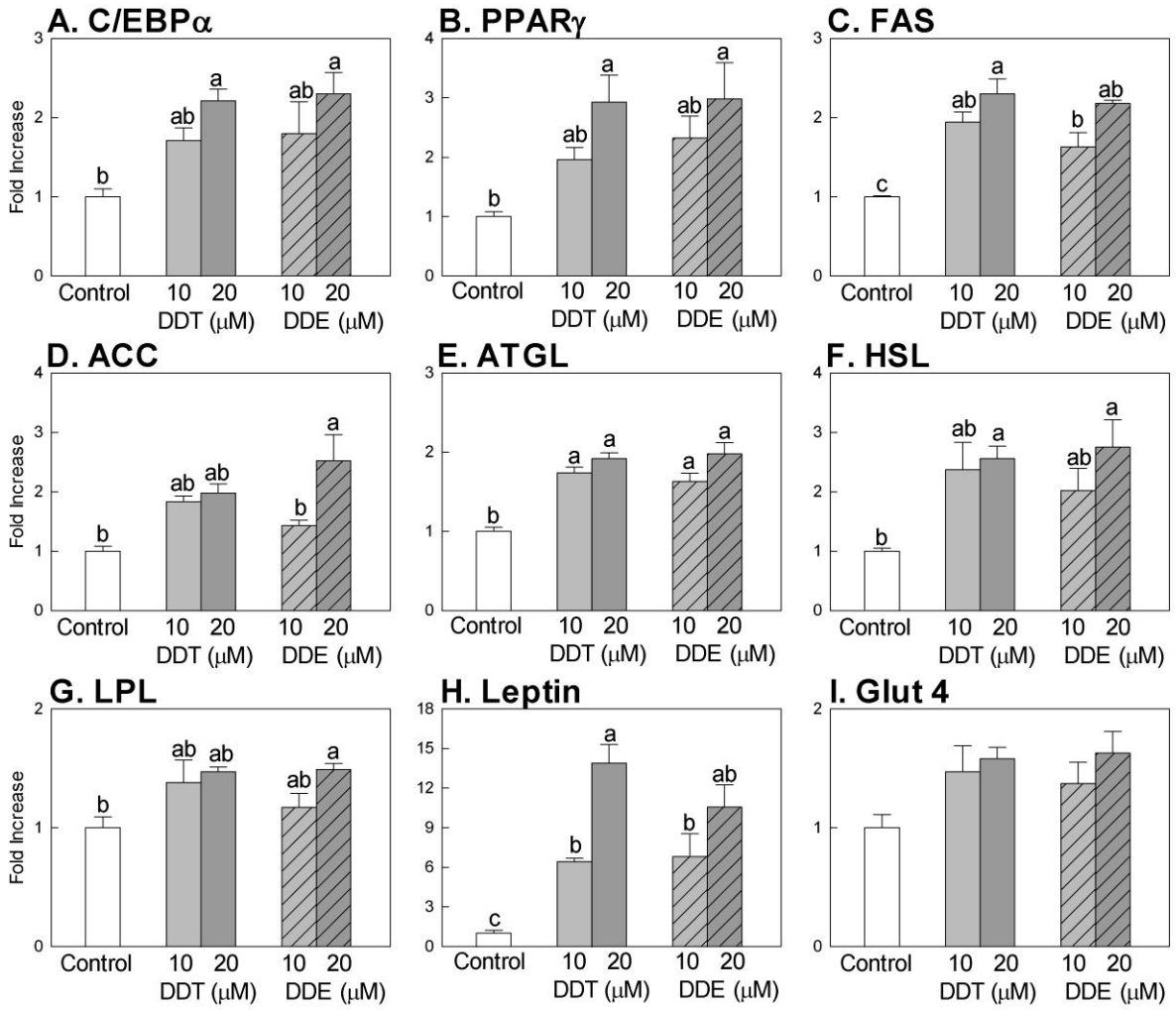


Figure 2

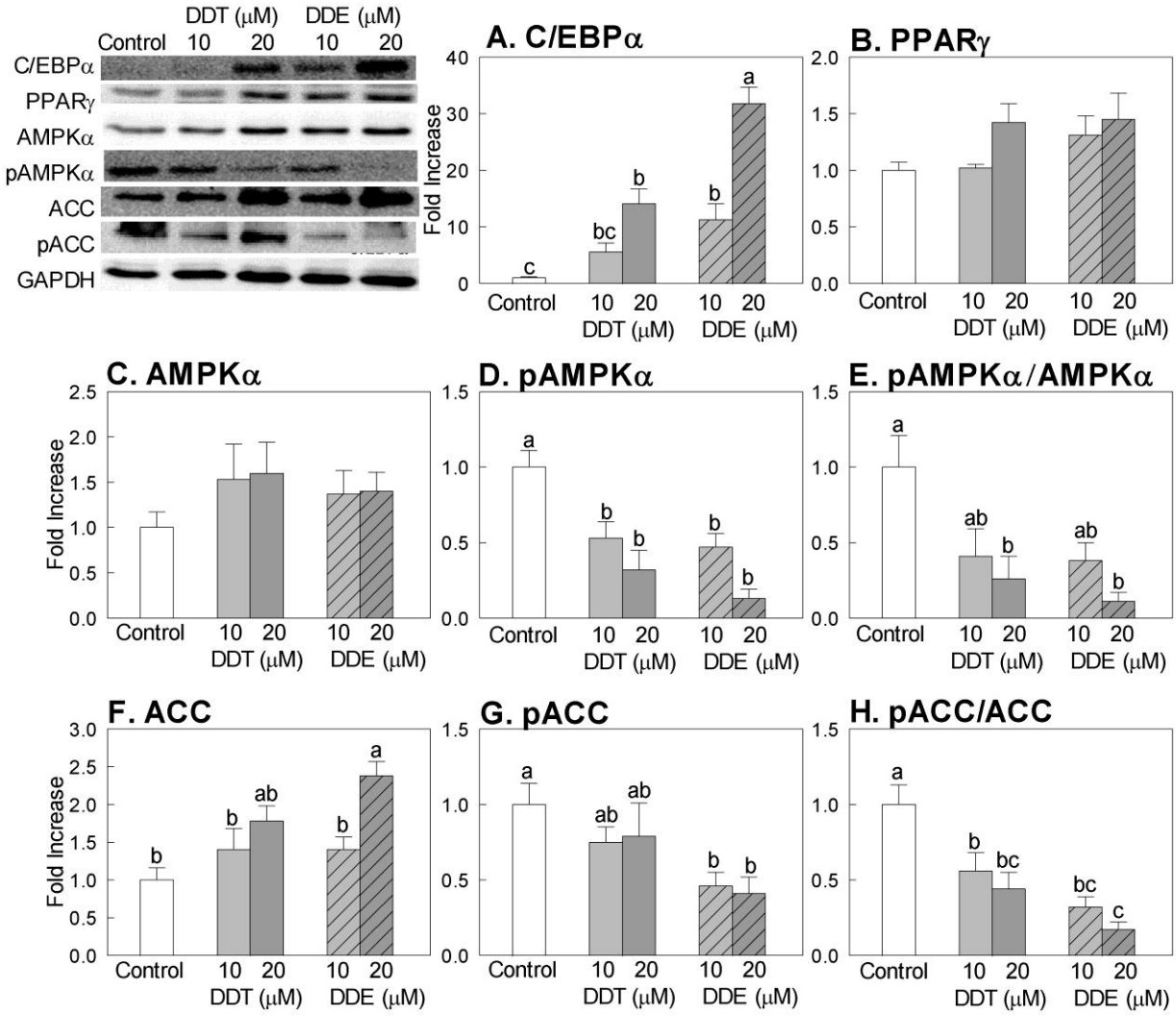
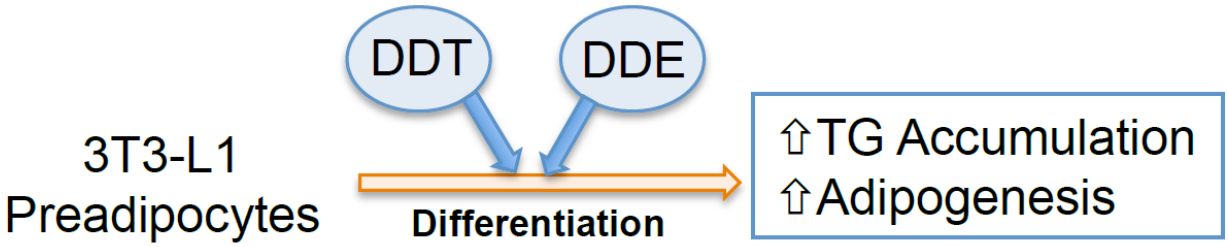


Figure 3



Graphical abstract

Highlights

- DDT and DDE potentiate lipid accumulation in 3T3-L1 adipocytes
- DDT and DDE stimulate adipocyte differentiation
- DDT and DDE potentiate adipogenesis via AMP-activated protein kinase-α