Published in final edited form as:

Toxicol Lett. 2015 October 14; 238(2): 65–71. doi:10.1016/j.toxlet.2015.07.009.

Exposure to p,p'-DDE enhances differentiation of 3T3-L1 preadipocytes in a model of sub-optimal differentiation

Lauren H. Mangum, George E. Howell III, Janice E. Chambers*

Center for Environmental Health Sciences, College of Veterinary Medicine, Mississippi State University, Mississippi State, MS, USA

Abstract

The incidence of obesity is increasing worldwide at an alarming rate. Recently, exposure to environmental contaminants, especially organochlorines such as p,p'-dichlorodiphenyldichloroethylene (DDE), has been implicated as a possible causative factor in the increasing obesity epidemic. The objective of this study was to evaluate the ability of DDE to alter adipogenesis in a model of sub-optimal differentiation. 3T3-L1 preadipocytes were induced to differentiate in the presence of DDE (0.01–100 μ M) using a sub-optimal differentiation cocktail. Eight days after the initiation of differentiation, adipogenesis was assessed through neutral lipid staining, triglyceride accumulation, and expression of markers of terminal differentiation. Exposure to DDE induced a concentration dependent increase in intracellular neutral lipid accumulation as determined by Oil Red O staining and triglyceride assay. Alterations in lipid accumulation were accompanied by upregulation of genetic markers of differentiation. DDE (10 μ M) enhanced expression of fatty acid binding protein 4 and Sterol regulatory element-binding protein-1c at the 2.5 and 20 μ M concentrations. DDE (2.5, 10, and 20 μ M) induced upregulation of leptin and fatty acid synthase, as compared to sub-optimal vehicle control (0.05% ethanol). Our results indicate that DDE is capable of enhancing adipogenesis and intracellular lipid accumulation in 3T3-L1 cells through upregulation of molecular targets responsible for lipid storage.

Keywords

3T3-L1; Adipogenesis; p,p'-DDE; Organochlorine insecticides

1. Introduction

The incidence of obesity and overweight has increased dramatically since the 1980's and is contributing to a major health crisis worldwide. Relative to normal weight, obesity is associated with increased relative risk of coronary artery disease, hypertension, insulin resistance, type 2 diabetes (T2D), all-cause mortality, and even some cancers (Hotamisligil,

*Corresponding author at: Center for Environmental Health Sciences, College of Veterinary Medicine, Mississippi State University, P.O. Box 6100, 240 Wise Center Drive, Mississippi State, MS 39762-6100, USA. Fax: +1 662 325 1031. chambers@cvm.msstate.edu (J.E. Chambers).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxlet.2015.07.009>.

2006; Ogden et al., 2014; Okwan-Duodu et al., 2013). The most commonly used metric for determining obesity is body mass index (BMI), or weight in kilograms divided by height in meters squared. More than two thirds (68.5%) of all adults in the United States are considered overweight or obese, defined as having a BMI $>25 \text{ kg/m}^2$ and $>30 \text{ kg/m}^2$, respectively, with more than one third (34.9%) of the population being obese (Ogden et al., 2014).

Adipose tissue has long been recognized as the storage depot for triglycerides, derived from excess nutritional energy, stored in lipid droplets inside white adipocytes in many different white adipose tissue depots. While these depots were once thought to be inert, adipose is now recognized as an active regulatory tissue that helps maintain lipid and glucose homeostasis (Guilherme et al., 2008). Obesity results from adipocyte hyperplasia and hypertrophy which leads to recruitment of immune cells to adipose tissue and eventually contributes to localized chronic low-grade inflammation and adipocyte dysfunction (McGinty and Young, 2011; Suganami and Ogawa, 2010). In the early stages of weight gain, excess energy is stored as lipid in mature adipocytes that expand in size. Afterward, there is a rapid increase in the number of fat cells as preadipocyte fibroblasts undergo proliferation and differentiation, a process called adipogenesis, to increase the number of mature fat cells (Lowe et al., 2011).

It is unlikely that lifestyle and dietary changes alone can account for the dramatic rise of overweight and obesity worldwide. Even animal populations that live in close contact with humans in industrialized nations have become prone to developing obesity. It has been estimated that the likelihood of this trend occurring purely by chance in animal populations at 1 in 12 million, indicating that an exogenous environmental factor is probably contributing to this development (Klimentidis et al., 2010). Recent research has suggested a strong association between chemical exposure and development of T2D and obesity (Lee et al., 2006; Longnecker and Daniels, 2001; Wang et al., 2010). These chemicals, described as “obesogens,” are suspected to contribute to the development of obesity by promoting lipid storage in mature adipocytes or by directly increasing the number of fat cells in adipose tissue (Li et al., 2012). One of the major groups of pesticides implicated in this research are the legacy organochlorine (OC) insecticides such as DDT and chlordane; these OC insecticides were used heavily during the 1950’s and ‘60’s, but have been banned in the US since the early 1970’s; however, legacy OC insecticides and their metabolites can still be found in serum samples and fatty substances, such as breast milk, in human populations both within the United States and abroad (Rylander et al., 2006). A number of cross sectional epidemiological studies have linked exposure to DDE with T2D and obesity. Recent human cohort studies have established an increased relative risk of developing T2D in individuals with the highest exposure levels (Lee et al., 2010, 2011). Additionally, recent *in vivo* exposure studies in rodents indicate that a high fat diet supplemented with persistent organic pollutants, including DDE, resulted in increased visceral adiposity and increased macrophage infiltration when compared to high fat diet alone (Ibrahim et al., 2011; Ruzzin et al., 2010). Due to its association with increased risk of obesity and obesity-related ailments, DDE exposure has been suggested as a potential biomarker for increased risk of disease, but its utility as a biomarker could be markedly enhanced if a mechanism were to be identified. As such, several *in vitro* studies have indicated a role for OC exposure and

enhanced adipogenesis and altered lipid metabolism in mature adipocytes. It has been reported that p,p'-DDT was able to enhance differentiation in 3T3-L1 or 3T3-F44A2 adipocytes (Moreno-Aliaga and Matsumura, 2002). Previous data from our lab found that exposure to DDE significantly increased basal fatty acid uptake and release of leptin, adiponectin, and resistin from mature adipocytes. However, in our previous study, DDE was unable to enhance adipogenesis during the differentiation of 3T3-L1 cells, possibly due to the use of a potent adipogenic cocktail that induced approximately 95% differentiation (Howell and Mangum, 2011).

In the present study, we furthered our previously published work examining the adipogenic effect of DDE exposure utilizing a sub-optimal differentiation cocktail and the previously utilized potent differentiation cocktail as a full differentiation, positive control. Through the use of the sub-optimal cocktail, we are now able to investigate the adipogenic potential of DDE in the absence of high background differentiation as performed in other studies examining the adipogenic effects of various compounds (Tung et al., 2014). Differentiating 3T3 cells were exposed to increasing concentrations of DDE in the presence of the sub-optimal and full strength adipogenic cocktail; then the accumulation of neutral intracellular lipid and triglyceride content was measured via Oil Red O (ORO) staining and commercial triglyceride assay, respectively. Finally, expression of markers and mediators of terminal adipocyte differentiation were assessed by two-step quantitative real-time polymerase chain reaction (RT-qPCR) to evaluate the potential mechanisms underlying any changes to adipogenesis observed in DDE treated cells.

2. Materials

Methylisobutylxanthine (IBMX), dexamethasone, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of IBMX (500 mM) were prepared in DMSO while dexamethasone was dissolved in ethanol (1 mg/ml) and diluted in 49 ml sterile medium. Solutions were aliquoted and stored at -20 °C. Triglyceride and MTT were from Cayman Chemical and Bradford reagent was from Bio-Rad Co. p,p'-DDE was purchased from ChemService (West Chester, PA).

3. Methods

3.1. 3T3-L1 culture and sub-optimal differentiation

Murine 3T3-L1 fibroblasts (American type culture collection, ATCC) were cultured in DMEM with 10% bovine calf serum (Sigma-Aldrich, St. Louis, MO) in a CO₂ incubator at 37 °C. For differentiation experiments, 3T3 cells were seeded onto multi-well tissue culture plates and grown to confluence. Two days post confluence, a sub-optimal differentiation protocol was initiated as previously described (Yan et al., 2003) and depicted in Fig. 1. Sub-optimal differentiation media, composed of 500 µM IBMX, 0.1 µM dexamethasone, and 167 nM human insulin, was added on the first day of differentiation (day 0). The media was exchanged 48 h later (day 2) for media containing 0.1 µM dexamethasone and 40 nM human insulin. DMEM containing 10% FBS (Sigma-Aldrich, St. Louis, MO) and 100 IU penicillin and 100 µg/ml streptomycin (Fisher Scientific) was used throughout the differentiation process (day 0-day 8). DDE (1.0 µM, 3.0 µM, 10 µM, 30 µM, 100 µM) was added on day 0

and maintained in culture until day 4. As a positive control, 3T3 cells were induced to differentiate in a full differentiation cocktail (FULL) consisting of 500 μ M IBMX, 1 μ M dexamethasone, and 10 μ g/ml human insulin followed by 10 μ g/ml human insulin in DMEM containing 10% FBS and 100 IU penicillin and 100 μ g/ml streptomycin (Fisher Scientific), for day 0 and day 2, respectively. Regardless of differentiation stimulus, during days 4–8, all differentiating 3T3 cells were maintained in DMEM with 10% FBS with media refreshed on day 6.

3.2. Cell viability

The effect of DDE on cell viability was determined by 3-(4,5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) based assay (Gerlier and Thomasset, 1986). Confluent 3T3 fibroblasts were incubated in media containing ethanol (vehicle, 0.05%) or DDE for 24 h. MTT reagent (10 μ l) was added to each well, followed by gentle agitation on an orbital shaker. Cells were incubated for an additional 4 h, media was carefully aspirated, and remaining formazan crystals were dissolved in 100 μ l of the provided crystal dissolving solution. Absorbance was measured at 570 nm according to manufacturer's directions (see Supplementary Fig. 1).

3.3. Assessment of intracellular neutral lipid accumulation by ORO staining

Eight days after the initiation of differentiation, adipogenesis was assessed using an ORO assay to measure intracellular neutral lipid content as previously described (Howell and Mangum, 2011). Briefly, on day 8 of differentiation, cells were washed with PBS, fixed in 10% formalin, and then stained for 10 min using ORO. Cells were rinsed with deionized water and allowed to air dry. Dye was extracted using 100% isopropanol and measured spectrophotometrically at 520 nm. Data are expressed as the fold change from sub-optimal differentiation vehicle due to varying efficiencies in the sub-optimal differentiation response.

3.4. Measurement of intracellular triglyceride

To ensure that the increase in ORO staining is due to increased intracellular triglyceride accumulation, 3T3 cells were differentiated as described above in the presence of vehicle, 10 or 20 μ M DDE. Following 8 days of differentiation, cells were scraped in triglyceride assay buffer (Cayman) and sonicated on ice. Triglyceride content of the supernatant was measured spectrophotometrically by reading the absorbance at 530 nm. Protein concentrations of the supernatant were measured by Bradford assay (Bio-rad) and triglyceride content (mg/dl) was normalized to protein (mg triglyceride/mg protein).

3.5. Gene expression analysis

3T3-L1 cells were seeded onto 12 well plates and maintained in DMEM with 10% CS or in a sub-optimal cocktail in the presence of vehicle (0.05% EtOH), and 2.5, 10, or 20 μ M DDE. To induce full differentiation, 3T3 cells were induced to differentiate in a full differentiation cocktail (FULL). Eight days after the initiation of differentiation, total RNA was extracted using Qiagen RNeasy Mini plus kit according to manufacturer's directions. RNA was quantified by NanoDrop and approximately 200 ng total RNA was subjected to cDNA synthesis using Maxima first strand cDNA synthesis kit (Thermo Scientific). Expression of

Fatty acid binding protein 4 (*Fabp4*), Peroxisome proliferator activated receptor- γ (*Pparg*), Fatty acid synthase (*Fasn*), Sterol regulatory elementbinding protein 1c (*Srebf1*), Glucose transporter type 4 (*Slc2a4*), and Leptin (*Lep*) were analyzed in 3T3-L1 cells. β -actin (*Actb*) was chosen as the reference gene for terminal differentiation (see Table 1 for sequence information) (Arsenijevic et al., 2012). Gene expression analysis by RT-qPCR was performed on a Stratagene MXP3005 using Taqman-based Luminaris Hgreen low ROX qRT-PCR reagents. Fold change in gene expression (2 individual wells/replicate; 3 pooled replicates total) was estimated using the $\Delta\Delta C_t$ method and statistical significance was determined as previously described (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

3.6. Statistical analysis

Data are expressed as mean with $\pm$ standard error (SE). Statistical significance was determined by one-way ANOVA with Duncan's Studentized range test for multiple comparisons using PROC GLM in SAS 9.3 (SAS Institute, Cary, NC).

4. Results

4.1. DDE enhances neutral lipid accumulation

Accumulation of intracellular neutral lipid was used as an indication of terminal differentiation in 3T3 cells. ORO staining of 3T3 cells differentiated in sub-optimal vehicle, DDE, or FULL indicated neutral lipid accumulation in all treatment groups, while ORO staining in 3T3-L1 cells maintained in DMEM with 10% CS was not significantly different from control (empty) wells. Fig. 2(A-C) shows lipid accumulation (red staining) in 3T3 cells differentiated in a sub-optimal cocktail in the presence of vehicle (A), 20 μ M DDE (B), compared with fully differentiated adipocytes (C). ORO staining indicated that, beginning at the 10 μ M concentration, DDE induced a concentration dependent increase in lipid accumulation compared to sub-optimal vehicle (Fig. 2D). The influence of DDE on 3T3 cell viability was assessed via serial dilution and measured by the MTT viability assay, however, only the highest concentration (100 μ M DDE) induced a slight but significant decrease in cell viability (see Supplementary Fig. 1).

4.2. DDE enhances triglyceride accumulation

ORO staining indicated a concentration-dependent increase in neutral lipid accumulation in response to DDE. To ensure that these lipids were triglyceride, a commercial triglyceride assay was employed to quantify lipid accumulation. DDE treatment increased triglyceride accumulation at both the 10 and 20 μ M concentrations by 42% and 58%, respectively, compared with sub-optimal vehicle control. Triglyceride accumulation was not significantly different between the two DDE treatment groups, but the 20 μ M concentration was significantly different from sub-optimal vehicle control. Triglyceride content was not measured in undifferentiated cells as it is not detectable by this assay and ORO staining revealed no neutral lipid accumulation after 8 days.

4.3. DDE increases expression of markers and mediators of adipogenesis

To investigate the potential mechanisms underlying the enhanced adipogenesis in 3T3 cells exposed to DDE under sub-optimal conditions of differentiation, expression levels of several genes involved in the adipogenic process were quantified by RT-qPCR. Compared to sub-optimal vehicle control, DDE exposure increased the expression of the transcription factor SREBP1c (encoded by *Srebp1c*), a mediator of adipocyte differentiation, as well as the expression of *Fasn*, a downstream target of SREBP1c and PPAR γ (encoded by *Pparg*), that is responsible for fatty acid biosynthesis and the coordination of intracellular triglyceride levels. All concentrations of DDE induced approximately 3 fold increase in *Srebp1c* expression while *Fasn* expression was doubled compared to sub-optimal vehicle. Additionally, DDE increased expression of *Fabp4* and *Lep*, two markers found in mature adipocytes. Expression of *Fabp4*, a lipid transporter protein, was increased by approximately 2 fold over sub-optimal vehicle while leptin, an adipokine that is partially responsible for controlling food intake, was increased by 5–7 fold compared to sub-optimal vehicle control (Fig. 3).

5. Discussion

Epidemiological data indicate that chemicals like DDE are associated not only with prevalence of abdominal obesity (Lee et al., 2012), which is a major risk factor for not only T2D, but other serious ailments like cancer and cardiovascular disease (Gustafson, 2010; Okwan-Duodu et al., 2013). Several *in vitro* studies have indicated a role for organochlorine pesticide exposure and enhanced adipogenesis and altered lipid metabolism in mature adipocytes. It has been reported that p,p'-DDT was able to enhance differentiation in 3T3-L1 or 3T3-F44A2 adipocytes (Moreno-Aliaga and Matsumura, 2002), while previous data from our lab found that exposure to DDE, the major bioaccumulative metabolite of DDT, significantly increased basal fatty acid uptake and release of leptin, adiponectin, and resistin from mature adipocytes; however, DDE was unable to enhance adipogenesis during the differentiation of 3T3-L1 cells, possibly due to the use of a potent adipogenic cocktail that induced approximately 95% differentiation (Howell and Mangum, 2011).

Our current findings are limited to an *in vitro* model utilizing concentrations representative of individuals falling within the 95th percentile of DDE serum concentrations (12.1 ng/ml or 38 nM; wet weight), as reported by the 2003–2004 NHANES study and were determined by utilizing the adipose to serum ratio as ~160:1 as reported by López-Carrillo et al., 1999 which would produce ~6 μ M DDE in the adipose. López-Carrillo et al., 1999 reported an average adipose tissue concentration calculated by adipose tissue wet weight of 1.6 μ M (515 ng/ml) with a maximum of 17.6 μ M (5613 ng/ml). While higher than median concentrations detected in the NHANES study, the DDE concentrations used in this *in vitro* work were physiologically relevant for those in the highest exposure range in the NHANES study and for concentrations reported by other studies (López-Carrillo et al., 1999). Our previous *in vitro* studies investigating the effect of DDE on adipogenesis were conducted under conditions that induce near complete adipogenesis using potent glucocorticoids and were unable to enhance adipogenesis as determined by ORO assay (Howell and Mangum, 2011). Since such potent conditions could mask any potential effect of DDE on differentiation,

3T3-L1 cells were differentiated under sub-optimal conditions in the present study. The results of this study indicated that DDE could enhance the accumulation of neutral lipids in a concentration dependent manner in 3T3 cells in a model of sub-optimal differentiation, as determined by semi-quantitative ORO staining. The observed increase in intracellular lipid staining was supported by the finding that DDE also increased triglyceride accumulation in these cells in a concentration dependent manner.

Previous studies from our lab (Howell and Mangum, 2011) investigated the potential for DDE exposure to perturb adipokine release from pre-differentiated, mature 3T3-L1 adipocytes. In contrast, the present study was intended to investigate the ability of DDE to enhance differentiation. It is important to note in this study that the DDE stimulus was removed 4 days prior to measurement of gene expression. The finding that DDE could increase 3T3 differentiation under sub-optimal conditions was supported by changes in expression of several adipogenic and lipogenic genes (Fig. 4). *Srebf1* is a transcription factor found in adipose tissue whose expression is induced following the induction of *Pparg*, and is responsible for the expression of several genes involved in glucose and lipid metabolism and homeostasis, such as *Fasn*, and thus facilitates the adipogenic program. While the induction of *Srebf1* was only significantly different from the sub-optimal vehicle at the 10 μ M DDE concentration, there was a positive trend seen with the 2.5 and 20 μ M concentrations as well. Fatty acid synthase (encoded by *Fasn*), an enzyme that is essential for the functional differentiation of adipocytes, is also regulated at the transcriptional level. Upregulation of *Fasn* during preadipocyte differentiation not only provides substrates for triacylglycerol synthesis, but these products are used to maintain and sustain signaling for the differentiation process. Furthermore, expression of *Fasn* is not only an endpoint in preadipocyte differentiation but is an active player (Schmid et al., 2005), so the upregulation of *Srebf1* and *Fasn* by DDE supports the findings of increased ORO staining and enhanced triglyceride accumulation.

Expression markers of terminal differentiation were also upregulated by DDE in comparison to sub-optimal vehicle control. Expression of *Fabp4*, a membrane associated protein that is thought to be involved in the uptake, transport, and metabolism of long chain fatty acids, was increased significantly by all concentrations of DDE. Additionally, in agreement with previously reported data from our lab (Howell and Mangum, 2011), expression of *Lep* was enhanced across all DDE doses, and was significantly increased compared to sub-optimal vehicle control (Fig. 4D). Secretion of leptin was not assessed in this study, but it has been shown that leptin secretion is coordinated with mRNA expression and previously reported data from our lab indicates that DDE exposure does increase secretion of the adipokine from mature 3T-L1 cells (Howell and Mangum, 2011; Park et al., 2013). Enhanced *Lep* expression supports the findings that DDE may be influencing differentiation through the enhancement of lipid uptake and storage since increased leptin signaling is associated with an increase in lipid accumulation in adipocytes. PPAR γ is a late-stage, lipid-activated, transcriptional factor that regulates genes involved in terminal adipocyte differentiation and lipid metabolism. Expression of *Pparg* is induced in early differentiation and gradually increases in expression over time. PPAR γ regulates the transcriptional activation of genes responsible for adipogenesis and lipid metabolism, including *Fabp4* and *Fasn* (Watanabe et al., 2003). The sub-optimal differentiation cocktail induced *Pparg* expression to the same

extent as the full differentiation cocktail, so it is not surprising that DDE did not enhance its expression compared to sub-optimal vehicle. Similarly, expression of *Slc2a4* was not significantly different in DDE or sub-optimal vehicle treated cells; however, expression was significantly higher in all sub-optimal and FULL differentiated cells compared to undifferentiated pre-adipocytes. *Slc2a4* is not expressed in preadipocytes, so its marked induction indicates that these cells were indeed adipocytes, not preadipocytes that were induced to take up lipid. Furthermore, *Slc2a4* expression is regulated by PPAR γ , whose expression was maximally induced by sub-optimal vehicle, DDE, and FULL treated cells. While there was a slight trend for an increase in *Pparg* expression in the DDE treated cells vs FULL or sub-optimal vehicle, the increases may not have been enough to induce drastic changes in *Slc2a4* expression (Eckertova et al., 2011).

6. Conclusions

The present findings provide evidence that DDE at high concentrations may be able to enhance differentiation. DDE induced significant increases in the expression of several genes related to adipogenesis and lipid metabolism. While exposure to OC compounds like DDE has been associated with increased risk of metabolic disease and T2D (Lee et al., 2011; Lind et al., 2013), there is no evidence of a direct causal relationship between exposure and development of disease. These data suggest that DDE may enhance both adipogenesis and lipid uptake and storage through altered gene expression, resulting in more adipocytes capable of accumulating lipid. This may predispose an individual with high exposure to developing increased visceral adiposity and result in increased weight gain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Center for Environmental Health Sciences and in part by the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health (NIH) under award number R15ES019742. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Abbreviations:

MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Actb	β -actin
BMI	body mass index
DDE	p,p'-dichlorodiphenyldichloroethylene
FABP4	fatty acid binding protein 4
Fasn	fatty acid synthase
Slc2a4	glucose transporter type 4

Lep	leptin
IBMX	methylisobutylxanthine
RT-qPCR	quantitative real-time polymerase chain reaction
ORO	Oil red O
OC	organochlorine
Pparg	peroxisome proliferator activated receptor- γ
Srebf1	sterol regulatory element-binding protein-1c
T2D	type 2 diabetes

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HIGHLIGHTS

- 3T3-L1 cells exposed to p,p'-DDE and differentiated under sub-optimal conditions.
- Lipid accumulation and expression of markers of differentiation were assessed.
- p,p'-DDE enhanced lipid and triglyceride accumulation.
- p,p'-DDE enhanced expression of markers and mediators of adipogenesis.

Fig. 1.
3T3-L1 exposure protocol. Sub-optimal differentiation protocol for 3T3-L1 cells.

Fig. 2. DDE enhances neutral lipid accumulation.

Representative photomicrograph of adipocytes differentiated using sub-optimal cocktail in the presence of vehicle (A), 20 μ M DDE (B) compared with fully differentiated adipocytes (C). Cells were stained with Oil Red O and dye was extracted and measured spectrophotometrically at 520 nm (D). Data ($n = 4-7$) are expressed as mean fold change compared to vehicle $\pm$ standard error (SEM). Statistical significance was determined by one-way ANOVA with Duncan's Studentized range test for multiple comparisons. Means with different letters indicate significant difference at $P < 0.05$.

Fig. 3. DDE enhances triglyceride accumulation.

Data ($n = 5$) are expressed as mean triglyceride (mg TG/mg protein) $\pm$ standard error (SEM). Triglyceride content was extracted and measured using commercial reagents. Statistical significance was determined by one-way ANOVA with Duncan's Studentized range test for multiple comparisons. Means with different letters indicate significant difference at $P < 0.05$.

Fig. 4. DDE upregulates mediators of differentiation.

Data ($n = 3$) are expressed as mean fold change compared to undifferentiated cells (CS) $\pm$ standard error (SEM). Differentiating 3T3 cells were exposed to increasing concentrations of DDE (2.5, 10, and 20 μ M), standard differentiation cocktail (FULL), or Vehicle (V; 0.05% ethanol). Markers and mediators of differentiation were measured by RT-qPCR. Statistical significance was determined by one-way ANOVA. Means with different symbols indicate significant difference at $P < 0.05$.

Table 1

Markers of terminal differentiation.

Gene name	Gene symbol	Primer sequence (5'–3')	Function
β Actin	Actb	F: GTCGAGTCGCGTCCACC R: GTCATCCATGGCGAACTGGT	Housekeeping
Peroxisome proliferator activated receptor- γ	Pparg	F: GAAAGACAAACGGACAAATCACC R: GGGGGTGATAATGTTTGAACCTTG	Mediators of differentiation
Sterol regulatory element-binding protein 1c	Srebf1	F: AAGCTGTCGGGGTAGCGTC R: GAGCTGGAGCATGCTCTCAA	Mediators of differentiation
Fatty acid synthase	Fasn	F: AGATGGAAGGCTGGGGCTCTA R: GAAGGCTCTCGGGATCTCTG	Mediators of differentiation
Leptin	Lep	F: CAGGATCAATGACATTTTCACACA R: GCTGGTGAGGACCTGTTGAT	Markers of terminal differentiation
Fatty acid binding protein 4	Fabp4	F: TGAAAGAAAGTGGGAGTGGGC R: CCAGCTTGTCACCAATCTCGT	Markers of terminal differentiation
Facilitated glucose transporter member 4	Slc2a4	F: GACGGACACTCCATCTGTGTG R: GCCACGATGGAGACATAGC	Markers of terminal differentiation