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Original Research Article

DDE downregulates PLIN2 expression during differentiation of mesenchymal stem cells into adipocytes in lipid-enriched medium



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ABSTRACT

Evidence indicating, that persistent organic pollutants are involved in the development of obesity, has emerged. The aim of this study was to reveal whether an environmental bioaccumulative human adipose tissue contaminant, 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE), affects adipocyte differentiation. Our study was conducted on an *in vitro* adipogenic model of human adipose derived mesenchymal stem cells (hADMSC). The adipose cultures were exposed to DDE (concentrations: 0.1 μ M, 1 μ M, and 10 μ M) for 28 consecutive days, from the beginning of the experiment until full differentiation. DDE was administered in lipid vehicle (NuTRiflex). Samples for gene expression analysis by RT real-time PCR were collected on days 0, 4, 10, 21 and 28 during the course of differentiation. Differentiating adipocytes cultivated in lipid-rich medium (NuTRiflex) increased the expression of perilipin 2 (PLIN2). However, the addition of DDE suppressed this effect ($p < 0.03$). Our results may suggest that upregulation of PLIN2, caused by exposure to lipids during the differentiation of adipocytes, is reduced in the presence of DDE. This effect of DDE warrants

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Abbreviations:DDE, *p,p'*-dichlorodiphenyldichloroethyleneDDT, *p,p'*-dichlorodiphenyltrichloroethane

POPs, persistent organic pollutants

LDs, lipid droplets

TG, triglyceride

PLIN2, perilipin 2

PKA, protein kinase A

future attention, because of the important role of PLIN2 in formation and stabilization of lipid droplets, as the impairment of their function could be linked to the worldwide obesity epidemic.

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Introduction

Many organochlorine compounds have been used worldwide as agricultural pesticides. Among those widely used was 1,1'-(2,2,2-trichloroethane-1,1-diyl)bis(4-chlorobenzene) (DDT) and although DDT has not been used in the developed countries since the 1970s, it is still present in the environment. DDE's chemical stability and extreme lipophilicity predispose it to exhibiting bioaccumulative properties similar to other persistent organic pollutants (POPs) with high affinity to adipose tissue, which represents their long-term reservoir. The presence of DDT, and perhaps more importantly that of its metabolic product 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE), is still detected in the food chain today (Maršálek et al., 2013). So it is by no means surprising to find POPs present in human tissues (Hardell et al., 2010; Waliszewski et al., 2012).

Recently, epidemiological studies have revealed that elevated serum concentrations of certain POPs, including DDE, correlate positively with increased type 2 diabetes prevalence in humans (Taylor et al., 2013). DDE's influence has been confirmed to be both diabetogenic and obesogenic (Dirinck et al., 2014). Studies of subacute exposure to DDE on mice models have demonstrated that DDE causes significant hyperglycaemia (Howell et al., 2014). The effect of chronic exposure to DDE in conjunction with a high fat diet was found to be biphasic; after initial promotion of fasting hyperglycaemia, glucose levels decreased and by week 13 animals chronically exposed to DDE were normoglycemic (Howell et al., 2015). Alongside their part in lipid metabolism, adipocytes may facilitate cancer progression by playing an important role in the tumor microenvironment (Omabe et al., 2015).

The specific mechanism of POPs' action is not yet known. POPs are almost exclusively stored within the lipid droplets (LDs) of adipocytes and they depend on triglyceride (TG) content (Bourez et al., 2012; Hong et al., 2012). LDs' size in adipocytes reflects the degree of their differentiation and the intensity of intracellular metabolic processes. The TG core of LDs is surrounded by phospholipids and a variety of proteins (Tauchi-Sato et al., 2002). One such protein is perilipin 2 (PLIN2), formerly known as adipose differentiation-related protein (ADRP). PLIN2 has long been recognized as a universal marker for intracellular LD in tissues, but its function is just

beginning to be elucidated. It has been discovered one of PLIN2's domains is involved in LD stabilization and lipid accumulation (Sentinelli et al., 2015). These findings are supported by the fact, that upregulation of PLIN2 is associated with TG storage in LDs. As adipocytes mature, they gain neutral lipids and PLIN2 is replaced by PLIN1 (Wolins et al., 2003, 2005).

In order to verify the hypothesis that DDE may influence adipocyte differentiation and metabolism, adipocytes, during their differentiation from human adipose derived mesenchymal stem cells (hADMSC), were exposed to DDE. Our study is the first to demonstrate that upregulation of PLIN2, caused by lipids during differentiation of hADMSC, was reduced by DDE.

Materials and methods

Cell culture and differentiation

For adipogenic differentiation, hADMSCs (Invitrogen Life Technologies GmbH, Darmstadt, Germany) were seeded with a total number of 1×10^5 cells in a 6-well cell culture plate (TPP Techno Plastic Products, Switzerland), and cultured in 5% CO₂ atmosphere at 37 °C according to the manufacturer's instructions in StemPro® Adipogenesis Differentiation medium supplemented with 1% Gentamicin.

Cell DDE treatment

The cultures were exposed to DDE (Sigma–Aldrich, St. Louis, MO, USA) for 28 consecutive days from the start of the experiment until full differentiation was achieved (DDE concentrations: 0.1 μM, 1 μM, and 10 μM). The concentrations of DDE were chosen to reflect the levels measured in humans (Dirinck et al., 2014). DDE was administered in the lipid fraction of NuTRiflex® Lipid peri (B. Braun, Melsungen, Germany), present in 0.2% (v/v) concentration (0.2 ml of vehicle per 100 ml of medium), containing medium chain triglycerides (0.1 g/l) and soya bean oil (0.1 g/l). Control cultures received the differentiation medium alone or with vehicle without DDE. The samples for quantitative estimation of mRNA were taken on days 0, 4, 10, 21 and 28. Fig. 1 shows the cell cultivation design used in the pilot trial and in the subsequent triplicate experiment.

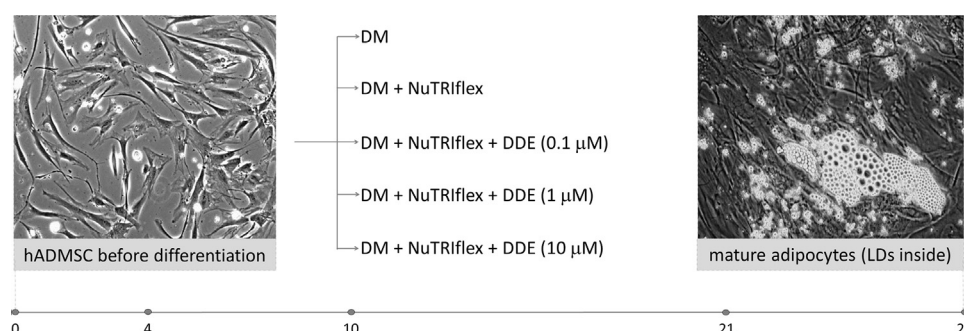


Fig. 1 – Cell cultures were divided into five sets: a set containing only DM; a set with DM supplemented with vehicle (NuTriflex) without DDE; and three sets containing DM and different concentrations of DDE (0.1 μ M, 1 μ M and 10 μ M) administered in vehicle. Round dots on the timeline indicate the days on which samples were taken. Abbreviations: DM, differentiation medium; DDE, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylen; hADMSC, human adipose derived mesenchymal stem cells; LDs, lipid droplets.

Table 1 – Sequences of primers and corresponding UPL probes.

Symbol	Gene name	Function	Primer sequence 5'–3'	UPL probe
PLIN2	Perilipin 2, ADRP	Surface protein of intracellular lipid droplets	TCAGCTCCATTCTACTGTTCACC CCTGAATTTTCTGATTGGCACT	72
<i>Reference genes</i>				
GUSB	β -Glucuronidase	Lysosomal breakdown of glycosaminoglycans	CGCCCTGCCTATCTGTATTC TCCCCACAGGGAGTGTGTAG	57
HPRT	Hypoxanthine guanine phosphoribosyltransferase	Purine salvage pathway	TGACCTTGATTTATTTGCATACC CGAGCAAGACGTTTCAGTCCT	73
YWHAZ	14-3-3 protein zeta	Signal transduction regulating adaptor	GCAATTACTGAGAGACAACTTGACA TGGAAGGCCGGTTAATTTT	2

Quantitative estimation of mRNA using RT real-time PCR

Quantitative estimation of the mRNA of PLIN2 was performed by a RT real-time PCR method with UPL probes (Universal Probe Library, Roche). Total RNA was isolated from a pellet of cells (approximately 10^5 cells) by FastRNAPro Green Kit (QBIogene, Irvine, CA, USA). Reverse transcription (RT) was performed from 250 ng of total RNA with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) using random hexamers as primers. The sequences of primers and corresponding UPL probes were generated by ProbeFinder Software (Roche, Mannheim, Germany) and are listed in Table 1.

A quantitative estimation was performed in technical duplicates on Stratagene Mx3005P apparatus (Agilent Technologies, Inc.). All samples were also assessed for the expression of reference genes β -glucuronidase (GUSB), hypoxanthine guanine phosphoribosyltransferase (HPRT) and tyrosine 3/tryptophan 5-monooxygenase activation protein zeta (YWHAZ). The results are presented as normalized values ($2^{-\Delta\Delta C_t}$ algorithm) using the geometric mean of quantifications (C_t) of three reference genes (Kozera and Rapacz, 2013).

Statistical analysis

Non-parametric Wilcoxon two-sided signed-rank test was performed for statistical analysis. The data is presented as

mean \pm standard error of mean. The results were considered statistically significant at the significance level $\alpha = 0.05$.

Results

Differentiating adipocytes cultivated in lipid-rich medium (NuTriflex) showed a statistically significant increase of PLIN2 expression in comparison with adipocytes cultivated in differentiation medium only. However, DDE appeared to diminish the effect of NuTriflex on PLIN2. Statistically significant downregulation of PLIN2 was recorded in cell cultures containing DDE at a concentration of 0.1 μ M and 1 μ M (using data from day 10 and day 21 together). Analysis of the change of expression of PLIN2 in cells exposed to DDE at a concentration of 10 μ M was statistically inconclusive. The presented results are summarized in Fig. 2.

Discussion

To the best of our knowledge, our study is the first to demonstrate that upregulation of PLIN2, caused by lipids during differentiation of hADMSC, was reduced by DDE. Although PLIN2's cellular function is not fully understood, it seems to be indispensable for intracellular lipid storage in a variety of cells. While PLIN2 upregulation in myocytes

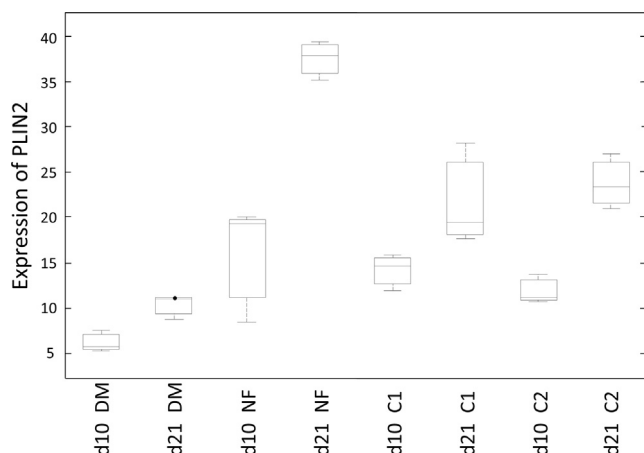


Fig. 2 – PLIN2 expression of differentiating cells cultivated in DM, DM and NuTRiflex, and DM, NuTRiflex and DDE (0.1 μ M, 1 μ M). Statistical analysis was conducted using the Wilcoxon two-sided signed-rank test using data from day 10 and day 21 together. Comparison between DM alone and DM with NuTRiflex was statistically significant as well as between DM with NuTRiflex and DM with DDE diluted in NuTRiflex at concentrations 0.1 μ M and 1 μ M.

Abbreviations: d10, day 10; d21, day 21; DM, differentiation medium; PLIN2, perilipin 2; NF, differentiation medium supplemented with NuTRiflex; C1, differentiation medium supplemented with NuTRiflex and DDE at concentration 1 μ M; C2 differentiation medium supplemented with NuTRiflex and DDE at concentration 0.1 μ M.

improves insulin resistance (Timmers et al., 2011), its over-expression in hepatocytes or endothelial cells leads to hepatic steatosis and the formation of foam plaques (Magnusson et al., 2006). It is suggested that PLIN2 plays an important role in the maturation of adipocytes (Storey et al., 2011). The molecular mechanism by which DDE changes PLIN2 expression has not been described yet. We suppose that the mechanism of DDE's effect could be mediated by the protein kinase A (PKA) signaling pathway. There is evidence that DDE decreases cAMP synthesis in stable pig granulosa cell lines (Crellin et al., 2001). The second messenger's, cAMP's, effect is mediated by PKA (Meinkoth et al., 1993). Liver X receptor (LXR) is one of the known target proteins phosphorylated by PKA (Yamamoto et al., 2007). Kotokorpi et al. have shown that the human PLIN2 gene is a direct LXR target gene and that different LXR agonists regulate the endogenous gene (Kotokorpi et al., 2010).

Furthermore, it has been confirmed that PLIN2 contains progesterone receptor binding sites (Yin et al., 2012). Progesterone partial agonist/antagonist RU486 upregulated PLIN2 expression in a dose- and time-dependent manner in breast cancer and uterine leiomyoma cells. Positive association between progesterone and PLIN2 levels was observed in endometrium during conceptus elongation as well (Forde et al., 2013). Therefore we suppose another way of affecting the expression of PLIN2 by DDE may be via the progesterone signaling pathway.

The results of our study suggest PLIN2 is downregulated by DDE during the differentiation of adipocytes exposed to lipids.

PLIN2 has an important role in the formation and stabilization of LDs, whose metabolism could be influenced by DDE in this way. This process warrants future attention, especially when the crucial role of LDs in energy homeostasis is considered, as the impairment of their function could be linked to the worldwide obesity epidemic and diabetes mellitus type 2. Better understanding the pathophysiological effects of the environmental pollutant DDE may help in the prevention of these diseases.

Conflict of interest

The authors declare no conflict of interest.

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