Journal of Hazardous Materials 182 (2010) 649–655

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on adipogenic differentiation and insulin-induced glucose uptake in 3T3-L1 cells

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ARTICLE INFO

Article history:
Received 22 January 2010
Received in revised form 2 June 2010
Accepted 19 June 2010
Available online 25 June 2010

Keywords:
TCDD
Adipogenesis
Glucose uptake
Insulin resistance
Adipocytes

ABSTRACT

Dioxin exposure has been positively associated with human type II diabetes. Because lipophilic dioxins accumulate mainly in adipose tissue, this study aimed to determine if dioxins induce metabolic dysfunction in fat cells. Using 3T3-L1 cells as an *in vitro* model, we analyzed the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a model dioxin, on adipogenic differentiation, glucose uptake, and lipolysis. TCDD inhibited adipogenic differentiation, as determined by using oil droplet formation and adipogenic marker gene expression, including PPARγ (peroxisome proliferator-activated receptor γ), C/EBPα (CCAAT/enhancer-binding protein α), and Glut4 (glucose transporter type 4). Effects of TCDD on glucose uptake were evaluated using fully differentiated 3T3-L1 adipocytes, revealing that TCDD significantly attenuated insulin-induced glucose uptake dose dependently. Inhibition of aryl hydrocarbon receptor (AhR) by α-naphthoflavone (α-NF), an AhR inhibitor, did not prevent the inhibitory effect of TCDD on glucose uptake, suggesting that TCDD attenuates insulin-induced glucose uptake in an AhR-independent manner. Effects of TCDD on lipolysis were determined using glycerol release assay. We found that TCDD had no marked effect on isoproterenol-induced glycerol release in fully differentiated 3T3-L1 adipocytes. These results provide *in vitro* evidence of TCDD's effects on fat cell metabolism, suggesting dioxin exposure in development of insulin resistance and type II diabetes.

1. Introduction

Dioxins are ubiquitous and highly toxic compounds that are byproducts of several chemical processes, such as incineration, combustion, and industrial manufacturing. The presence of dioxins in environment occurs mainly as a result of anthropogenic sources [1]. Dioxins are lipophilic, have a half-life between 7 and 9 years, and tend to accumulate in human bodies along the food chain [2,3]. Because of the unique nature of dioxins, it is important to understand their possible impact on human health.

It has been reported that dioxin exposure results in developmental toxicity, immune effects, endometriosis, cancer, diabetes, and cardiovascular disease in humans [4,5]. The symptom of a loss of adipose tissue and body mass, along with elevated serum lipid profiles, is known as wasting syndrome. A similar change in lipid profiles has also been found in dioxin-exposed humans [6,7]. Among dioxins, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic congener. Studies in laboratory animals – including guinea pigs [8,9], mice, rats [10,11], and monkeys [12] – indicated that TCDD exposure interfered with nutritional homeostasis by reducing adipose tissue mass, resulting in altered serum lipid profiles such as hyperlipidemia and hypertriglyceridemia [11,12]. TCDD is belongs in the category of persistent organic pollutants, which are lipophilic and easily trapped in adipose tissue. Adipocytes play a critical role in regulation of food intake, energy balance, and metabolic homeostasis via production of a number of endocrine hormones, i.e., adipokines. Animal studies have shown that mice subjected to a single high dose of TCDD resulted in inhibition of adipocyte differentiation [13,14]. Failure of adipocytes to adequately sequester excess lipids, resulting in their redistribution to other organs and tissues, appears to be a key event in the development of obesity-related metabolic dysfunction.

The present study aims to investigate the effects of TCDD on differentiation and fat/glucose metabolism of fat cells using a well-established *in vitro* adipocyte model: mouse 3T3-L1 cells. In this study, we have provided *in vitro* evidence indicating a possible linkage of TCDD exposure with the inhibition of insulin-induced glucose uptake in matured 3T3-L1 adipocytes.

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doi:10.1016/j.jhazmat.2010.06.081

2. Materials and methods

2.1. Chemicals and cells

TCDD (48599) was purchased from Supelco (Bellefonte, PA, USA). DL-Isoproterenol hydrochloride (I5627), α-naphthoflavone (α-NF) (N5757), insulin (I6634), 3-isobutyl-1-methylxanthine (IBMX, I5879), dexamethason (D4902), and Oil Red O (O0625) were purchased from Sigma (St. Louis, MO, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (475989) was purchased from Calbiochem (La Jolla, CA, USA). Glycerol assay kits were purchased from Randox (Randox Lab., Co., Antrim, UK). 2-[1-14C]-Deoxy-D-glucose ([14C]2-DOG) (NEC495A050UC) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). Mouse 3T3-L1 cells were purchased from the Bioresources Collection and Research Center of the Food Industry Research and Development Institute (Hsinchu, Taiwan) and were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine serum in a humidified atmosphere containing 5% CO_2 at 37 °C. 3T3-L1 cells were established through clonal isolation from 3T3 cells (Swiss albino), originated from disaggregated mouse embryos. The cells undergo a pre-adipose to adipose like conversion as they progress from a rapidly dividing to a confluent and contact inhibited state. The cells are sensitive to lipogenic hormones (e.g., epinephrine and insulin) and lipolytic hormones (e.g., isoproterenol) [15].

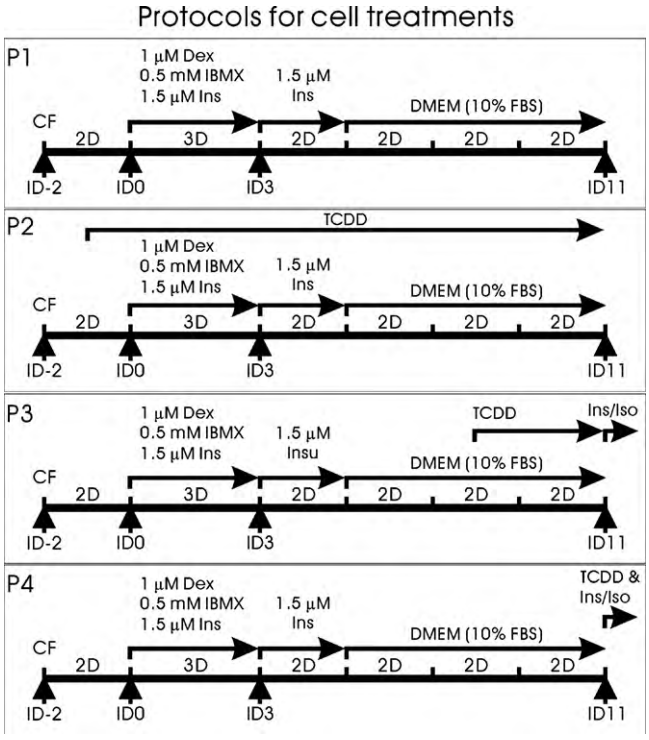

Fig. 1. Protocols for cell treatments in this study. The standard procedure of adipogenic differentiation is shown in P1. 3T3-L1 cells were cultured to confluence (CF). Two days postconfluence (ID0) the cells were incubated in adipogenesis-inducing medium (AIM) (DMEM containing 1 μM dexamethason (Dex), 0.5 mM IBMX, 1.5 μM insulin (Ins), and 10% FBS) for 3 days (ID3), then in adipogenesis-maintaining medium (AMM) (DMEM containing 1.5 μM insulin and 10% FBS) for 2 days (ID5), followed by DMEM with 10% FBS for another 6 days (ID11). The medium was changed every 2–3 days as indicated. To define the effects of TCDD on adipogenic differentiation (P2), the cells were treated with TCDD from ID-1 to ID11. Two TCDD treatments (P3 and P4) were used for analysis of glucose uptake and glycerol release. For P3 protocol, the cells were subjected to the standard procedure of adipogenic differentiation. At ID8, the cells were treated with TCDD for 3 days. At ID11, the treated cells were used for analysis of glucose uptake or glycerol release. For P4 protocol, 3T3-L1 cells were subjected to the standard procedure of adipogenic differentiation. At ID11, the cells were used for analysis of glucose uptake or glycerol release in the presence of TCDD. Iso, isoproterenol.

Table 1
PCR primers used in this study.

Gene	Forward primers (5′ → 3′)	Reverse primers (5′ → 3′)
PPARγ	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGAGAT
C/EBPα	TTACAACAGGCCAGGTTTCC	CTCTGGGATGGATCGATTGT
Glut4	GATTCTGCTGCCCTTCTGTC	ATTGGACGCTCTCTCTCCAA
β-Actin	ACACCCCAGCCATGTACG	TGGTGGTGAAGCTGTAGCC

2.2. Cytotoxicity assay

To determine the cytotoxic effects of TCDD, undifferentiated 3T3-L1 cells were seeded on 96-well plates at a density of 1×10^4 cells/well and then were incubated overnight. The cells were treated with various concentrations of TCDD (0.1, 1, 10, and 30 nM) for 24 h. After treatments, relative survival rates were determined using the MTT cytotoxicity assay as previously described in detail [16].

2.3. Induction of adipogenesis

The procedure for induction of adipogenesis of 3T3-L1 cells was performed as previously described in detail [17] with minor modifications. As shown in Fig. 1 (P1), 2 days postconfluence (ID0), the cells were incubated in adipogenesis-inducing medium (AIM) (DMEM containing 1 μM dexamethason, 0.5 mM IBMX, 1.5 μM insulin, and 10% FBS) for 3 days (ID3), then in adipogenesis-maintaining medium (AMM) (DMEM containing 1.5 μM insulin and 10% FBS) for 2 days (ID5), followed by DMEM with 10% FBS for another 6 days (ID11). The medium was changed every 2–3 days. At ID11, more than 95% of cells were fully differentiated into adipocytes.

2.4. Analysis of oil droplet formation during adipogenesis using Oil Red O staining

Oil Red O solution was prepared by dissolving 0.36% of Oil Red O in 60% isopropanol and then filtered with a 0.45-μm filter (Millipore, Molsheim, France). After treatments, cells on 6-well plates were washed with phosphate-buffered saline (PBS) twice and then were stained using the Oil Red O solution for 15 min at room temperature. Dye was extracted from cells on culture dishes with 3 ml of dye extraction solution (4% NP-40 in 60% isopropanol) and was quantified on a spectrophotometer with absorbance at 520 nm.

2.5. Quantification of adipogenic marker gene expression using qPCR

3T3-L1 cells were pretreated with TCDD (30 nM) for 24 h and then were subjected to the standard procedure of adipogenesis induction in the presence of TCDD, as shown in Fig. 1 (P2). At ID0, ID3, ID5, and ID10, total RNA samples were isolated from 3T3-L1 cells using the High Pure RNA Isolation Kits (Roche Diagnostics GmbH, Mannheim, Germany). DNA-free total RNA (1.0 μg) was reverse transcribed using MMLV reverse transcriptase (200 units) (Promega, Madison, WI, USA), RNasin (20 units) (Promega), and oligo(dT)$_{18}$ (1 μg) in a final volume of 20 μl. The sequences of mouse-specific primer sets for qPCR quantification of three adipogenic marker genes and the β-actin gene are listed in Table 1. Quantitative measurement of cDNA using the LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostics GmbH) was performed with a LightCycler instrument (Roche Diagnostics GmbH) according to the manufacturer's instructions. To confirm

the amplification specificity, the PCR products were subjected to melting curve analysis. Gene expressions were relatively quantified by the calibration against standard curves generated with a serial dilution of the first-strand cDNA mix. For quantification, data were analyzed with LightCycler analysis software according to the manufacturer's instructions. All qPCR assays were performed at least in triplicate. The adipogenic marker genes were normalized with β-actin.

2.6. TCDD treatments

Two different TCDD treatments (P3 and P4, shown in Fig. 1) were used for analysis of glucose uptake assay and glycerol release assay. Following the P3 protocol, 3T3-L1 cells were subjected to the standard procedure of adipogenesis induction. At ID8, the cells were treated with TCDD (0.1, 1, and 10 nM) for 3 days. At ID11, the treated cells were used for analysis of glucose uptake assay or glycerol release assay. Following the P4 protocol, 3T3-L1 cells were subjected to the standard procedure of adipogenesis induction. At ID11, the differentiated cells were used for analysis of glucose uptake assay or glycerol release assay in the presence of TCDD (0.1, 1, and 10 nM).

2.7. Glucose uptake assay

Glucose uptake assay was determined as previously described [18,19] with modifications. After treatments, cells were washed twice with 37 °C Krebs–Ringer phosphate (KRP) buffer (pH 7.4) (128 mM NaCl, 4.7 mM KCl, 1.65 mM $CaCl_2$, 2.5 mM $MgSO_4$, and 5 mM Na_2HPO_4). Cells were either left untreated or treated with insulin (100 nM) for 10 min in KRP buffer. Without changing the buffer, glucose uptake was started by addition of 2-[1-^{14}C]-deoxy-D-glucose ([^{14}C]2-DOG) (0.1 μCi/well) for an additional 10 min at 37 °C. Cells were gently washed three times with ice-cold DPBS and lysed in an 800 μl solution containing 0.5 M NaOH and 0.1% SDS. Sample were assayed for [^{14}C]2-DOG uptake using a Topcount NXT Scintillation Counter (Packard Instrument Company, Meriden, CT, USA). The level of glucose uptake induced by insulin (100 nM) was set as 100%.

2.8. Glycerol release assay

Lipolysis in differentiated 3T3-L1 adipocytes was analyzed using glycerol release assay as previously described in detail [20], with minor modifications. After treatments, glycerol released in culture medium was measured by a colorimetric method using glycerol assay kits (Randox Laboratories Ltd., Antrim, UK) following the manufacturer's instructions.

2.9. Statistical analysis

All qualitative data are representative of at least three independent experiments. Quantitative data are presented as means ± SD. The statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Scheff post hoc test using the Statistical Package for the Social Science 13.0 software (SPSS, Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. TCDD causes no marked cytotoxic effect on 3T3-L1 cells

To determine the cytotoxic effect of TCDD, undifferentiated 3T3-L1 preadipocytes were left untreated or treated with different concentrations of TCDD (0.1, 1, 10, or 30 nM) for 24 h. After treatments, cell viability was measured using MTT assay. As shown in Fig. 2, treatments with TCDD ≤ 30 nM caused no marked cytotoxicity to the undifferentiated 3T3-L1 preadipocytes, indicating that undifferentiated adipocytes were relatively resistant to TCDD treatments.

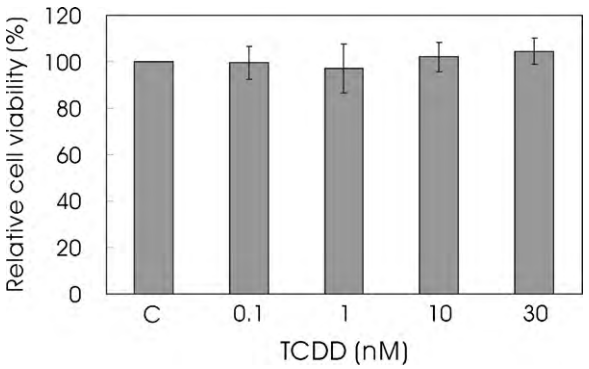

Fig. 2. TCDD had no marked cytotoxic effects on 3T3-L1 cells. Undifferentiated 3T3-L1 cells were treated with different concentrations of TCDD (0.1, 1, 10, and 30 nM) for 24 h. After treatments, cytotoxicity was analyzed using MTT assay. Cell viability was normalized with that of untreated control (C, DMSO solvent control) and is shown as percentages of relative cell viability. Data are presented as means ± SD ($n = 3$).

3.2. TCDD inhibits the adipogenic differentiation

To examine the effect of TCDD on adipogenic differentiation, 3T3-L1 cells were induced to adipogenesis in the presence of different concentration of TCDD (0.1, 1, 10, or 30 nM) according to the P2 protocol shown in Fig. 1. At ID11, the levels of oil droplet formation in 3T3-L1 adipocytes were evaluated using the Oil Red O staining. As shown in Fig. 3, treatments with 0.1, 1, 10, and 30 nM TCDD resulted in decreases in oil droplet formation by 18% ($p < 0.001$), 14% ($p < 0.001$), 20% ($p < 0.001$), and 22% ($p < 0.001$), respectively.

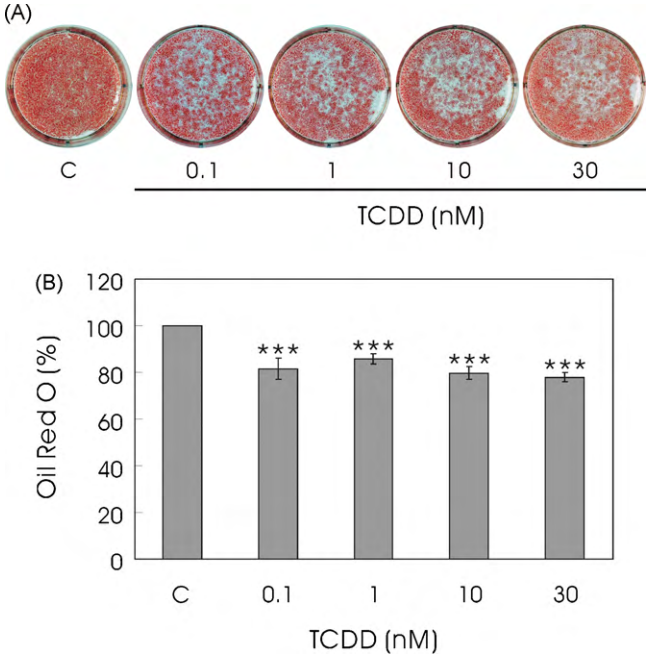

Fig. 3. TCDD inhibits adipogenic differentiation. To define the effects of TCDD on adipogenic differentiation, 3T3-L1 cells were left untreated (C, DMSO solvent control) or treated with TCDD (0.1, 1, 10, and 30 nM) from ID-1 to ID11 following the P2 protocol shown in Fig. 1. After treatments, (A) images of Oil Red O-stained adipocytes on plates were taken. (B) Levels of adipogenesis were determined by quantification of Oil Red O staining. Data are presented as means ± SD ($n = 3$). $^{***}p < 0.001$ versus the untreated control (C).

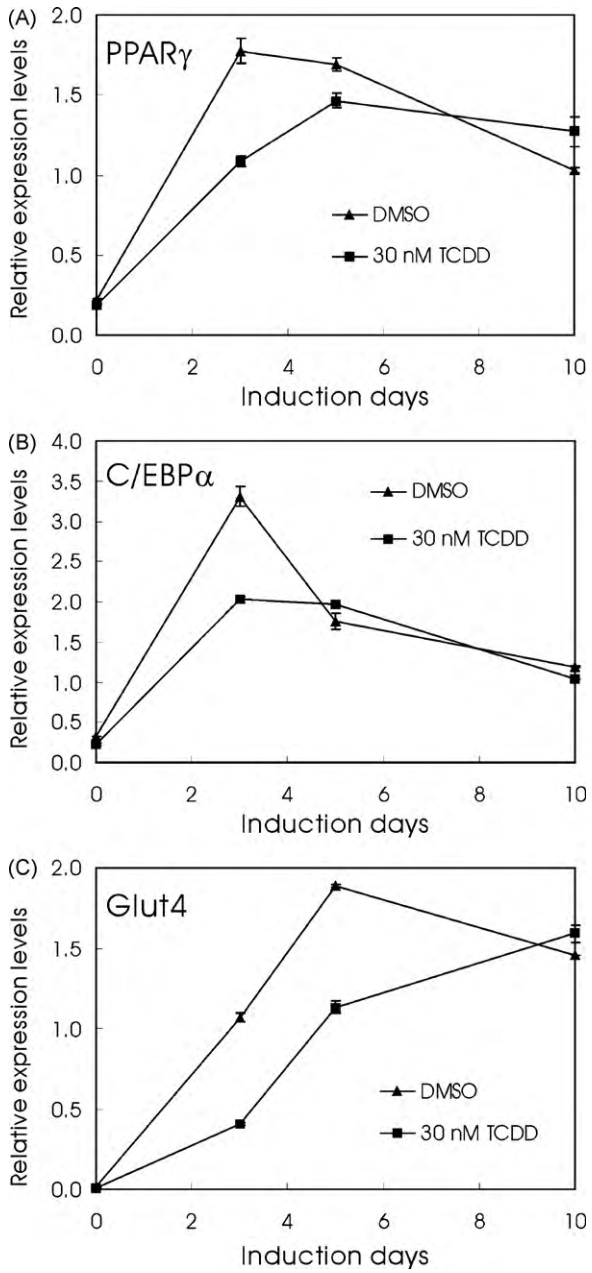

Fig. 4. Adipogenic marker gene expression was determined using qPCR. 3T3-L1 cells were left untreated (DMSO, solvent control) or treated with TCDD (30 nM) following the P2 protocol shown in Fig. 1. At ID0, ID3, ID5, and ID10, total RNA samples were isolated from 3T3-L1 At day 0, 3, 5 and 10 post-induction, total RNA samples were collected and expression of adipogenic marker genes PPARγ, C/EBPα, and Glut4, was quantified using qPCR. The adipogenic marker genes were normalized with β-actin. Data are presented as means ± SD ($n = 3$).

Using microscopy, we found that the decreased levels of Oil Red O staining by the TCDD treatments were due to the reduced percentage of fully differentiated adipocytes. Meanwhile, 30 nM TCDD caused no marked cytotoxic effect on the cells.

Moreover, we examined the effects of TCDD on expression of three adipogenic marker genes – i.e., PPARγ (peroxisome proliferator-activated receptor γ), C/EBPα (CCAAT/enhancer-binding protein α), and Glut4 (glucose transporter type 4) – in 3T3-L1 cells during adipogenic differentiation using qPCR. The cells were induced to adipogenesis in the presence of 30 nM TCDD and then mRNA samples were collected at ID0 (right before the hormonal induction), ID3 (after the hormonal induction for 3 days), ID5 (after the insulin treatment for 2 days), and ID10 (one day before full adipogenic differentiation) (P2 protocol, Fig. 1). As shown in Fig. 4, data from untreated controls (DMSO solvent controls) revealed the time-course changes of those three marker genes during adipogenic differentiation of 3T3-L1 cells. At the ID3, the TCDD treatment caused inhibition of gene expression of PPARγ, C/EBPα, and Glut4 by 44%, 43%, and 63%, respectively. At ID5, the TCDD treatment caused inhibition of gene expression of PPARγ and Glut4 by 15% and 40%, respectively. At the ID10, the TCDD treatment caused no marked effect on expression of those three marker genes. These results indicate that TCDD inhibits those adipogenic marker gene expressions at the early stage in adipogenic differentiation of 3T3-L1 cells.

3.3. TCDD attenuates the insulin-induced glucose uptake in an AhR-independent manner

Insulin-induced glucose uptake by adipose tissue plays a critical role in the maintenance of whole-body glucose homeostasis. The involvement of TCDD in interfering with glucose uptake by adipocytes may lead to insulin resistance and disruption of glucose homeostasis. Using isotope-labeled glucose, [14C]2-DOG, the effects of TCDD on glucose uptake by differentiated 3T3-L1 adipocytes were determined. First, the system was validated by an approximately 4–5-fold increase in glucose uptake induced by 100 nM of insulin (Fig. 5). Two different treatment protocols (P3 and P4, Fig. 1) were used in this study. In P3 protocol, the cells were pre-treated with TCDD (0.1, 1, or 10 nM) for 3 days (form ID8 to ID11), which was followed by glucose uptake assay induced by insulin (100 nM) in the absence of TCDD. Our results showed that the TCDD treatments caused no marked effect on the insulin-induced glucose uptake (Fig. 5A). In P4 protocol, without pre-treated with TCDD, the cells were treated with insulin (100 nM) in the presence of TCDD at ID11. Our results showed that treatments with 0.1, 1, and 10 nM TCDD inhibited the insulin-induced glucose uptake by 12%, 31% ($p < 0.05$), and 44% ($p < 0.001$), respectively (Fig. 5B). These results indicate that direct TCDD treatments attenuate the insulin-induced glucose uptake by adipocytes, suggesting a potential diabetogenic role of TCDD.

TCDD causes multiple systemic and cellular effects mainly via activation of AhR [21]. An AhR inhibitor α-NF was used to determine if TCDD attenuated the insulin-induced glucose uptake via activation of AhR. As shown in Fig. 5C, treatment with α-NF ≤10 nM was not able to recover the inhibitory effect of TCDD on insulin-induced glucose uptake. Meanwhile, it was noted that cytotoxicity induced by 10 μM α-NF resulted in a further inhibition of insulin-induced glucose uptake by 71% ($p < 0.01$). Our results indicate that TCDD attenuates the insulin-induced glucose uptake in an AhR-independent manner.

3.4. TCDD causes no marked effect on the isoproterenol-induced glycerol release

Adenylyl cyclase/cAMP-dependent pathway is one of the major mechanisms by which lipolysis is activated in adipocytes [22]. Activation of β-adrenoceptors by catecholamines leads to an increased intracellular cAMP production by adenylyl cyclase, which is followed by the activation of protein kinase A and hormone-sensitive lipase, the major enzyme involved in lipolysis [23]. To determine the effect of TCDD on lipolysis, we conducted a quantitative *in vitro* measurement of glycerol released from differentiated 3T3-L1 adipocytes into culture medium using the glycerol release assay. First, the system was validated by an approximately 2-fold increase in glycerol release induced by 1 μM of isoproterenol, a β-adrenergic agonist (Fig. 6). Again, two different treatment protocols (P3 and P4, Fig. 1) were used in this study. Our results show that nei-

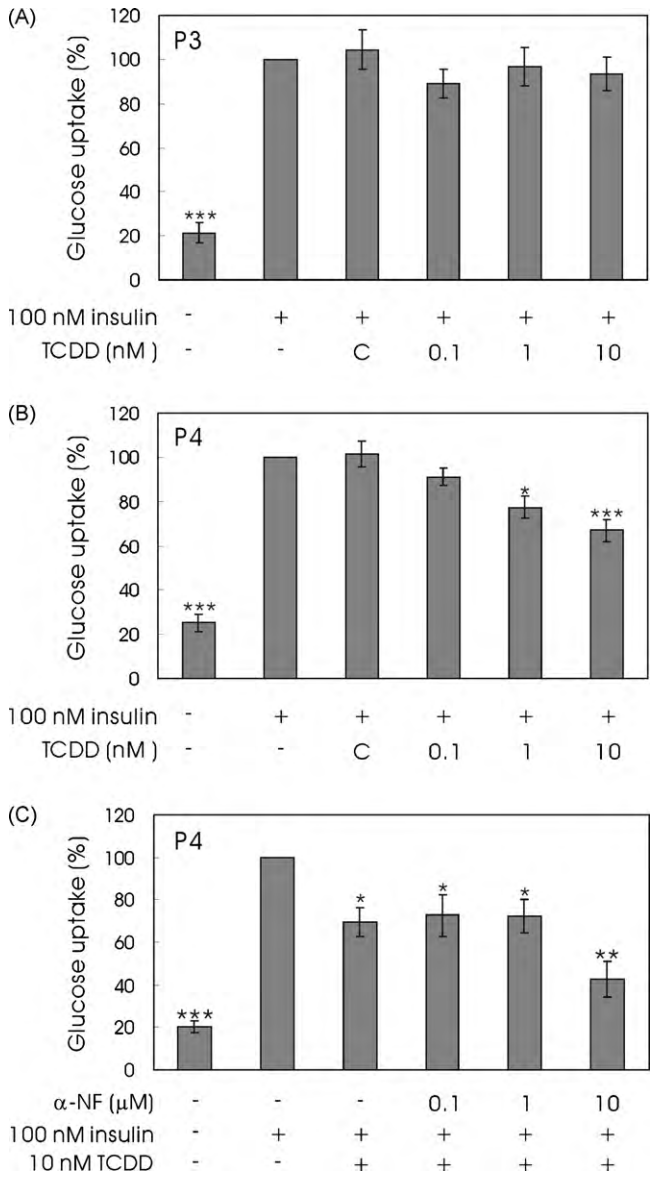

Fig. 5. TCDD inhibits the insulin-induced glucose uptake in an AhR-independent manner. Two TCDD treatments (P3 and P4) were used for analysis of glucose uptake by 3T3-L1 cells. (A) For P3 protocol, the cells were subjected to the standard procedure of adipogenic differentiation. At ID8, the cells were left untreated (C, DMSO solvent control) or treated with TCDD (0.1, 1, and 10 nM) for 3 days. At ID11, the treated cells were used for analysis of glucose uptake. (B) For P4 protocol, the cells were subjected to the standard procedure of adipogenic differentiation. At ID11, the cells were used for analysis of glucose uptake in the presence of TCDD (0.1, 1, and 10 nM). The level of glucose uptake induced by insulin (100 nM) was set as 100%. Data are presented as means ± SD ($n = 7$). $^{*}p < 0.05$ and $^{***}p < 0.001$ versus treatments with insulin alone. (C) The cells were treated following the P4 protocol, except that before subjected to the TCDD (10 nM) and insulin (100 nM) treatment the cells were pretreated with α-NF (0.1, 1, 10 μM) for 4 h. Data are presented as means ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus treatments with insulin alone.

ther pretreatments (P3) nor co-treatments (P4) with TCDD cause any significant effect on the isoproterenol-induced glycerol release from fully differentiated 3T3-L1 adipocytes (Fig. 6).

4. Discussion

In this study we evaluated the molecular mechanism underlying the TCDD-induced metabolism dysfunction in fat cells using a well-established *in vitro* model, mouse 3T3-L1 cells. The adipogenic program in preadipocytes cell lines consists of several sequential

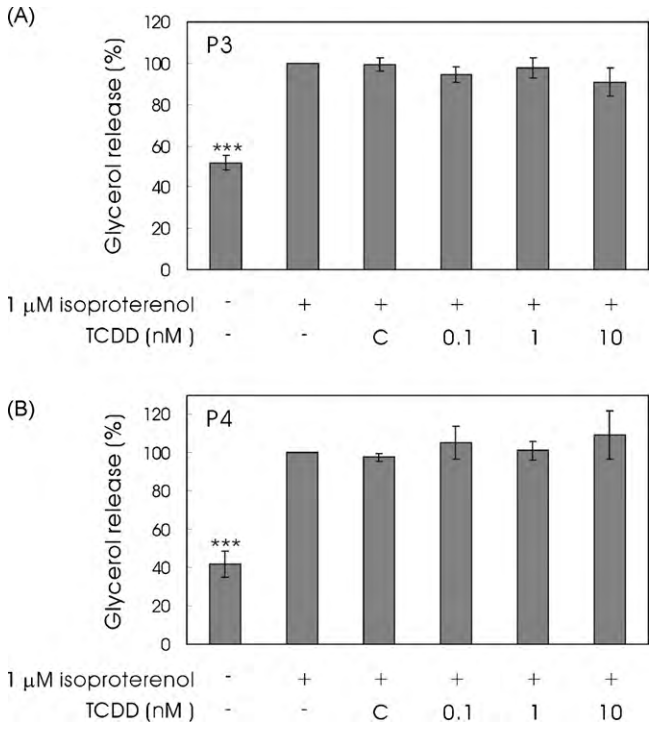

Fig. 6. TCDD causes no marked effect on the isoproterenol-induced glycerol release. Two TCDD treatments (P3 and P4) were used for analysis of glycerol release from 3T3-L1 cells. (A) For P3 protocol, the cells were subjected to the standard procedure of adipogenic differentiation. At ID8, the cells were left untreated (C, DMSO solvent control) or treated with TCDD (0.1, 1, and 10 nM) for 3 days. At ID11, the treated cells were used for analysis of glycerol release. (B) For P4 protocol, the cells were subjected to the standard procedure of adipogenic differentiation. At ID11, the cells were used for analysis of glycerol release in the presence of TCDD (0.1, 1, and 10 nM). The level of glycerol release induced by isoproterenol (1 μM) was set as 100%. Data are presented as means ± SD ($n = 7$). $^{***}p < 0.001$ versus treatments with isoproterenol alone.

steps, i.e., determination of a preadipocyte fate, growth arrest at confluence, clonal expansion, growth arrest, and terminal differentiation [24]. The transcriptional control of adipogenesis involves the activation of several families of transcription factors, which are temporally expressed in different steps during adipogenesis [24]. Previous studies in 3T3-L1 cells [25] and C3H/10T1/2 cells [26] indicated that TCDD treatments prior to hormonal induction of adipogenesis were critical for the effective inhibition of adipogenesis. Therefore, it would be critical to select the timing of TCDD treatments regarding the temporal expression of those genes potentially targeted by TCDD. Protocols for cell treatments in this study are shown in Fig. 1. To define the effects of TCDD on adipogenic differentiation, we treated the cells with non-toxic concentrations of TCDD (0.1, 1, 10, and 30 nM) (Fig. 2) 1 day before the induction of differentiation (ID-1) through day 11 of differentiation (ID11). Our mRNA samples for qPCR analysis of time-course changes in gene expression were collected from cells in four critical steps in adipogenic differentiation: ID0 (right before the hormonal induction), ID3 (after the hormonal induction for 3 days), ID5 (after the insulin treatment for 2 days), and ID10 (one day before full adipogenic differentiation).

Our results clearly demonstrated that the TCDD treatments significantly attenuated adipogenic differentiation of 3T3-L1 cells using oil droplet formation (Fig. 3). Moreover, results from qPCR revealed that the TCDD treatment caused marked decreases in gene expression of PPARγ, C/EBPα, and Glut4 after the hormonal induction for 3 days (ID3) and that this inhibition was getting weaker (for PPARγ) or not observed (for C/EBPα) when the hormonal induction was replaced with the insulin treatment (ID5)

(Fig. 4). It is well known that TCDD-induced cellular responses are mainly mediated through activation of AhR [21]. Upon full adipogenic differentiation, transcription levels of AhR in 3T3-L1 cells are reduced markedly [27,28]. Effects of TCDD on inhibition of adipogenic differentiation involve decreased expression of PPARγ, a key player in adipogenesis [26], and have been demonstrated to be AhR-dependent [14,25]. Previous studies have shown that PPARγ and C/EBPα cross-regulate each other to maintain their gene expression and also regulate expression of other adipogenic marker genes – including aP2, Glut4, and LPL – during adipogenic differentiation [29,30]. On the basis of these findings, we hypothesize that TCDD inhibits expression of PPARγ and/or C/EBPα as well as their downstream adipogenic differentiation via AhR. Meanwhile, decreasing expression of AhR during adipogenic differentiation makes the cells insensitive to TCDD treatments. It was also noted that, in the presence of insulin (ID3 and ID5), the TCDD treatment caused a marked decrease in Glut4 expression and this inhibition was not detected when insulin was removed from the culture medium (ID10) (Fig. 4C), suggesting the possible involvement of TCDD in interfering with insulin-mediated signals. Because of the critical role of Glut4 in regulation of the insulin-induced signals in adipocytes, TCDD-perturbed adipogenic differentiation during sensitive periods, such as fetal or early childhood development, may contribute to the later development of hyperglycemia or glucose intolerance.

Indeed, association of TCDD exposure and increased incidences of type II diabetes [31–36] and insulin resistance [37] has been demonstrated in epidemiological studies. Animal studies also have revealed that TCDD exposure inhibits insulin-induced glucose uptake [38] as well as impairs the second phase of glucose-stimulated secretion of insulin from islets [39] in mice via the AhR-dependent signals. Previous *in vitro* studies showed that TCDD significantly reduced the basal [40] as well as the insulin-induced glucose uptake [41] by differentiated 3T3-L1 adipocytes. Furthermore, in the present study, we demonstrated that TCDD, as in combined treatments with insulin, significantly inhibits the insulin-induced glucose uptake by fully differentiated 3T3-L1 adipocytes in a dose-dependent manner (Fig. 5B). However, this inhibitory effect was not observed in those TCDD-pretreated cells (Fig. 5A). In general, these studies support the idea that insulin resistance and glucose intolerance resulted from the TCDD-induced adipocyte dysfunction may play a critical role in the development of type II diabetes. Because transcription levels of AhR in 3T3-L1 cells are reduced markedly upon full adipogenic differentiation [27,28], it is of importance to determine if AhR plays any role in the TCDD-induced inhibition of glucose uptake shown in Fig. 5B. By using an AhR inhibitor α-NF, we found that TCDD inhibited the insulin-induced glucose uptake by 3T3-L1 adipocytes in an AhR-independent pathway (Fig. 5C). Because TCDD is readily accumulated in adipose tissue, these studies provide a possible physiological mechanism for epidemiological studies that link dioxin to type II diabetes.

Studies in humans [6,7] and various animals [8,9,11,12] have revealed the association between TCDD exposure and the lipid irregularity. Lipolysis is a key factor in the adverse metabolic consequences observed in subjects with higher levels of visceral fat accumulation [42]. However, it was difficult to demonstrate the TCDD-induced lipolysis *in vitro* using mature 3T3-L1 adipocytes, possibly due to their limited AhR expression. An optimum cell culture condition using 3T3-L1 adipocytes for studying the action of TCDD on lipolysis has recently been proposed, in which the optimum condition was found to require 7-day differentiated adipocytes being subjected to DMEM medium containing TCDD (but without insulin) for 5 days incubation with two medium changes on incubation days 2 and 4 [41]. The study showed that incubation of the cells with TCDD (10 nM) for 5 days resulted in significant signs of lipolytic changes, i.e., decreases of several marker genes, including PPARγ, C/EBPα, LPL, Glut4, and IRS-1 [41]. In the present study, we determined to further ask, in addition to the "lipolysis-like phenomenon", if "real lipolysis" could be detected in the adipocytes subjected to TCDD treatments. Using glycerol release assay for measuring cellular breakdown of triacylglyceride, our results indicated that TCDD causes no marked effect on the isoproterenol-induced glycerol release in differentiated 3T3-L1 adipocytes (Fig. 6). However, it was noted that TCDD treatments during adipogenic differentiation induced an increased production of TNFα [43] as well as a decreased expression of LPL [41,43]. Our experiment protocols could not exclude the possibility that, as combined with those autocrine inflammatory cytokines such as TNFα, TCDD may promote lipolysis in adipocytes.

5. Conclusions

Using 3T3-L1 cells, we have demonstrated that TCDD (1) inhibits adipogenic differentiation, (2) attenuates the insulin-induced glucose uptake in an AhR-independent manner, and (3) causes no marked effect on the isoproterenol-induced lipolysis. These results provide *in vitro* evidence for the TCDD effects on fat cell metabolism, suggesting the possible involvement of dioxin exposure in the development of insulin resistance or type II diabetes. Because 3T3-L1 cells, originally established from mouse embryos, are not used to address the differences between fat cells from different tissues or organs, further studies are needed to determine whether the insulin resistance and type II diabetes associated with TCDD reflects a general effect on adipocytes or a specific effect on visceral fat cells.

Acknowledgments

We are grateful to Mr. Mark Swofford for critical editing of the manuscript and to Professor Yung-hsi Kao (Department of Life Science, National Central University, Taiwan) and Professor Chi-Chang Juan (Institutes of Physiology and Clinical Medicine, National Yang-Ming University, Taiwan) for their kind help. This work was supported by grants from the National Science Council (NSC97-2314-B-400-003-MY3) and the National Health Research Institutes (EO-097-PP-07 and EO-98-PP-05) in Taiwan.

References

[1] P.S. Kulkarni, J.G. Crespo, C.A. Afonso, Dioxins sources and current remediation technologies—a review, Environ. Int. 34 (2008) 139–153.
[2] H.J. Geyer, I. Scheunert, K. Rapp, I. Gebefugi, C. Steinberg, A. Kettrup, The relevance of fat content in toxicity of lipophilic chemicals to terrestrial animals with special reference to dieldrin and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), Ecotoxicol. Environ. Saf. 26 (1993) 45–60.
[3] J.E. Michalek, J.L. Pirkle, S.P. Caudill, R.C. Tripathi, D.G. Patterson Jr., L.L. Needham, Pharmacokinetics of TCDD in veterans of Operation Ranch Hand: 10-year follow-up, J. Toxicol. Environ. Health 47 (1996) 209–220.
[4] EFSA, Dioxins: Methodologies and Principles for Setting Tolerable Intake Levels for Dioxins, Furans and Dioxin-like PCBs, European Food Safety Authority (EFSA), Brussels, Belgium, 2004.
[5] D. Mullerova, J. Kopecky, White adipose tissue: storage and effector site for environmental pollutants, Physiol. Res. 56 (2007) 375–381.
[6] A.E. Walker, J.V. Martin, Lipid profiles in dioxin-exposed workers, Lancet 1 (1979) 446–447.
[7] J.V. Martin, Lipid abnormalities in workers exposed to dioxin, Br. J. Ind. Med. 41 (1984) 254–256.
[8] T.A. Gasiewicz, R.A. Neal, 2,3,7,8-Tetrachlorodibenzo-p-dioxin tissue distribution, excretion, and effects on clinical chemical parameters in guinea pigs, Toxicol. Appl. Pharmacol. 51 (1979) 329–339.
[9] D.W. Brewster, F. Matsumura, TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) reduces lipoprotein lipase activity in the adipose tissue of the guinea pig, Biochem. Biophys. Res. Commun. 122 (1984) 810–817.
[10] R.A. Neal, P.W. Beatty, T.A. Gasiewicz, Studies of the mechanisms of toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), Ann. N. Y. Acad. Sci. 320 (1979) 204–213.
[11] B.N. Gupta, J.G. Vos, J.A. Moore, J.G. Zinkl, B.C. Bullock, Pathologic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals, Environ. Health Perspect. 5 (1973) 125–140.

[12] E.E. McConnell, J.A. Moore, D.W. Dalgard, Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rhesus monkeys (Macaca mulatta) following a single oral dose, Toxicol. Appl. Pharmacol. 43 (1978) 175–187.
[13] A.E. Brodie, V.A. Azarenko, C.Y. Hu, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) inhibition of fat cell differentiation, Toxicol. Lett. 84 (1996) 55–59.
[14] D.L. Alexander, L.G. Ganem, P. Fernandez-Salguero, F. Gonzalez, C.R. Jefcoate, Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis, J. Cell Sci. 111 (Pt 22) (1998) 3311–3322.
[15] H. Green, O. Kehinde, An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion, Cell 5 (1975) 19–27.
[16] T.C. Tsou, S.C. Yeh, F.Y. Tsai, L.W. Chang, The protective role of intracellular GSH status in the arsenite-induced vascular endothelial dysfunction, Chem. Res. Toxicol. 17 (2004) 208–217.
[17] J.S. May, A.M. Prince, R.E. Lyle, R.E. McGehee Jr., Antisense suppression of p107 inhibits 3T3-L1 adipocyte differentiation, Biochem. Biophys. Res. Commun. 283 (2001) 837–842.
[18] D.S. Paul, A.W. Harmon, C.P. Winston, Y.M. Patel, Calpain facilitates GLUT4 vesicle translocation during insulin-stimulated glucose uptake in adipocytes, Biochem. J. 376 (2003) 625–632.
[19] A.W. Harmon, D.S. Paul, Y.M. Patel, MEK inhibitors impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes, Am. J. Physiol. Endocrinol. Metab. 287 (2004) E758–766.
[20] C.C. Juan, C.L. Chang, Y.H. Lai, L.T. Ho, Endothelin-1 induces lipolysis in 3T3-L1 adipocytes, Am. J. Physiol. Endocrinol. Metab. 288 (2005) E1146–1152.
[21] J. Mimura, Y. Fujii-Kuriyama, Functional role of AhR in the expression of toxic effects by TCDD, Biochim. Biophys. Acta 1619 (2003) 263–268.
[22] G.Y. Carmen, S.M. Victor, Signalling mechanisms regulating lipolysis, Cell Signal. 18 (2006) 401–408.
[23] D. Langin, Adipose tissue lipolysis as a metabolic pathway to define pharmacological strategies against obesity and the metabolic syndrome, Pharmacol. Res. 53 (2006) 482–491.
[24] S.M. Rangwala, M.A. Lazar, Transcriptional control of adipogenesis, Annu. Rev. Nutr. 20 (2000) 535–559.
[25] M. Phillips, E. Enan, P.C. Liu, F. Matsumura, Inhibition of 3T3-L1 adipose differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin, J. Cell Sci. 108 (Pt 1) (1995) 395–402.
[26] M.A. Cimafranca, P.R. Hanlon, C.R. Jefcoate, TCDD administration after the pro-adipogenic differentiation stimulus inhibits PPARgamma through a MEK-dependent process but less effectively suppresses adipogenesis, Toxicol. Appl. Pharmacol. 196 (2004) 156–168.
[27] S. Shimba, K. Todoroki, T. Aoyagi, M. Tezuka, Depletion of arylhydrocarbon receptor during adipose differentiation in 3T3-L1 cells, Biochem. Biophys. Res. Commun. 249 (1998) 131–137.
[28] S. Shimba, M. Hayashi, T. Ohno, M. Tezuka, Transcriptional regulation of the AhR gene during adipose differentiation, Biol. Pharm. Bull. 26 (2003) 1266–1271.
[29] F.M. Gregoire, C.M. Smas, H.S. Sul, Understanding adipocyte differentiation, Physiol. Rev. 78 (1998) 783–809.
[30] E.D. Rosen, B.M. Spiegelman, Molecular regulation of adipogenesis, Annu. Rev. Cell Dev. Biol. 16 (2000) 145–171.
[31] G.L. Henriksen, N.S. Ketchum, J.E. Michalek, J.A. Swaby, Serum dioxin and diabetes mellitus in veterans of Operation Ranch Hand, Epidemiology 8 (1997) 252–258.
[32] P.A. Bertazzi, I. Bernucci, G. Brambilla, D. Consonni, A.C. Pesatori, The Seveso studies on early and long-term effects of dioxin exposure: a review, Environ. Health Perspect. 106 (Suppl. 2) (1998) 625–633.
[33] J. Vena, P. Boffetta, H. Becher, T. Benn, H.B. Bueno-de-Mesquita, D. Coggon, D. Colin, D. Flesch-Janys, L. Green, T. Kauppinen, M. Littorin, E. Lynge, J.D. Mathews, M. Neuberger, N. Pearce, A.C. Pesatori, R. Saracci, K. Steenland, M. Kogevinas, Exposure to dioxin and nonneoplastic mortality in the expanded IARC international cohort study of phenoxy herbicide and chlorophenol production workers and sprayers, Environ. Health Perspect. 106 (Suppl. 2) (1998) 645–653.
[34] J.E. Michalek, F.Z. Akhtar, J.L. Kiel, Serum dioxin, insulin, fasting glucose, and sex hormone-binding globulin in veterans of Operation Ranch Hand, J. Clin. Endocrinol. Metab. 84 (1999) 1540–1543.
[35] P.T. Fujiyoshi, J.E. Michalek, F. Matsumura, Molecular epidemiologic evidence for diabetogenic effects of dioxin exposure in U.S. Air force veterans of the Vietnam war, Environ. Health Perspect. 114 (2006) 1677–1683.
[36] S.L. Wang, P.C. Tsai, C.Y. Yang, Y. Leon Guo, Increased risk of diabetes and polychlorinated biphenyls and dioxins: a 24-year follow-up study of the Yucheng cohort, Diabetes Care 31 (2008) 1574–1579.
[37] J.W. Chang, H.L. Chen, H.J. Su, P.C. Liao, H.R. Guo, C.C. Lee, Dioxin exposure and insulin resistance in Taiwanese living near a highly contaminated area, Epidemiology 21 (2010) 56–61.
[38] P.C. Liu, F. Matsumura, Differential effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the "adipose-type" and "brain-type" glucose transporters in mice, Mol. Pharmacol. 47 (1995) 65–73.
[39] H. Kurita, W. Yoshioka, N. Nishimura, N. Kubota, T. Kadowaki, C. Tohyama, Aryl hydrocarbon receptor-mediated effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on glucose-stimulated insulin secretion in mice, J. Appl. Toxicol. 29 (2009) 689–694.
[40] H. Olsen, E. Enan, F. Matsumura, Regulation of glucose transport in the NIH 3T3 L1 preadipocyte cell line by TCDD, Environ. Health Perspect. 102 (1994) 454–458.
[41] W. Li, C.F. Vogel, F. Matsumura, Studies on the cell treatment conditions to elicit lipolytic responses from 3T3-L1 adipocytes to TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin, J. Cell Biochem. 102 (2007) 389–402.
[42] P. Arner, Human fat cell lipolysis: biochemistry, regulation and clinical role, Best Pract. Res. Clin. Endocrinol. Metab. 19 (2005) 471–482.
[43] P.A. Kern, A. Dicker-Brown, S.T. Said, R. Kennedy, V.A. Fonseca, The stimulation of tumor necrosis factor and inhibition of glucose transport and lipoprotein lipase in adipose cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin, Metabolism 51 (2002) 65–68.