UPPSALA
UNIVERSITET

An investigation of the effects of dibutyl phthalate (DBP) exposure on insulin sensitivity in C2C12 cells

Xue Yu

Master's Degree Project in Medical Research, 30 credits, Spring 2022
Department: Department of Surgical Sciences, Uppsala University
Supervisor: Wen Liu

Table of Contents

Abstract

Background

Dibutyl phthalate (DBP) is widely used as a plasticizer in a variety of products, including food packaging, personal-care products, and medical devices. Epidemiological studies have associated circulating levels of DBP with metabolic diseases, including increased risk for type 2 diabetes (T2D). However, the molecular link between DBP exposure and increased risk for T2D is still unknown. Herein, this study aims to investigate the effects of DBP on insulin sensitivity in skeletal muscle cells.

Aim

To study the effects of DBP on insulin sensitivity, cell viability, and GLUT4 membrane translocation in skeletal muscle cells.

Methods/Materials

Mouse myoblast cell line C2C12 was used as the skeletal muscle cell model in this study. C2C12 cells were treated with different concentrations of DBP (0.1 nM, 1 nM, 10 nM, 100 nM, and 1 μM), and 0.1% DMSO treated C2C12 cells were used as the control. Insulin sensitivity of C2C12 cells was measured by insulin-stimulated uptake of fluorescently labeled D-glucose analog, 2-(N-[7-nitrobenz-2-oxa-1, 3-diazol-4-yl] amino)-2-deoxyglucose (2-NBDG), using flow cytometry. The cytotoxic effect of DBP on C2C12 cells was evaluated by the cell viability assay. Glucose transporter type 4 (GLUT4) distribution in C2C12 cells treated with 10nM or 100 nM DBP was measured by immunofluorescence staining.

Results

Insulin-stimulated 2-NBDG uptake in C2C12 cells treated with DBP (10 nM, 100 nM, and 1 μM) significantly decreased. The cell viability assay showed no significant difference in cell viability among DBP exposure (0.1 nM, 1 nM, 10 nM, 100 nM, and 1 μM) group and the control. Immunofluorescence staining results showed that membrane translocation of GLUT4 was decreased in C2C12 cells treated with 10 nM and 100 nM DBP.

Conclusions

DBP exposure (10 nM, 100 nM, and 1 μM) impairs insulin sensitivity in C2C12 cells, and it is not caused by the cytotoxicity of DBP. DBP (10 nM and 100 nM) treated cells might exhibit a decreased level of GLUT4 translocated on the plasma membrane in response to insulin.

Keywords

DBP, insulin sensitivity, GLUT4, skeletal muscle

Popular science summary

This chemical leads to skeletal muscle insulin resistance, and it is hard to avoid

Insulin resistance is when cells in your muscles, brain, fat, and liver respond poorly to insulin and cannot take up glucose for energy. The ability of skeletal muscle cells to take up glucose when stimulated with insulin is crucial for glucose homeostasis. Failure to do so will lead to an abnormally high glucose level in the blood, which promotes the development of many diseases, including obesity, hypercholesterolemia, and type 2 diabetes. Risk factors, such as sedentary lifestyle, high fat or carbohydrate diet, smoking, and insomnia, have been identified. Recently, increasing attention has been drawn to a group of chemical compounds in many consumer products: phthalates.

Phthalates and plastics often appear hand in hand. Due to their ability to increase the flexibility of plastics, phthalates are commonly used as plasticizers in food packaging, children's toys, home furnishing, and personal care products. However, phthalates have been increasingly receiving negative publicity due to their detrimental impact on public health. Epidemiology studies have associated phthalates with infertility and metabolic disorders. In the present study, we investigated the effects of exposure to dibutyl phthalate (DBP) on cultured mouse skeletal muscle cells and found that DBP was harmful to the insulin sensitivity of skeletal muscles.

We tested the effects of different concentrations of DBP, ranging from 0.1 nM to 1 μM, on cultured mouse skeletal muscle cells. We found that when stimulated by insulin, cells treated with 10 nM, 100 nM, and 1 μM DBP did not uptake as much glucose as they normally do. We further performed the cell viability test to see whether this impaired insulin sensitivity could be caused by the cytotoxicity of DBP. The results showed that exposing to DBP did not affect cell viability. To better understand how DBP impairs skeletal muscle insulin sensitivity, we analyzed a protein called glucose transporter type 4 (GLUT4). GLUT4 rests within the cells in the basal condition and will insert into the cell plasma membrane and transport glucose into skeletal muscle cells in response to insulin stimulation. We found that less GLUT4 was expressed on the cell plasma membrane in cells exposed to 10 nM and 100 nM DBP, suggesting DBP might interfere with insulin-stimulated GLUT4 membrane translocation.

Studies on environmental pollutants have shown that the daily exposure level of DBP is around 213 nM, which according to our study, might induce insulin resistance in skeletal muscles in the human body. However, when DBP enters our body, it can be decomposed into metabolites, such as mono-(3-carboxypropyl) phthalate (MCPP). Whether it will enhance or weaken DBP's effects on skeletal muscle cells is still unknown. Therefore, more research needs to be done on how DBP affects living animals. Additionally, we hope this study will ring the alarm on the use of phthalates in consumer products and encourage more effort to put into eliminating phthalates from our environment.

Introduction

Phthalates

Phthalates are phthalic acid esters widely used in the polymer industry since the 1930s (Gao & Wen, 2016). The ability to impart elasticity and durability of plastics makes them an ideal additive to numerous plastic materials, including polyvinyl chloride (PVC). Thus, it is not surprising that phthalates are commonly applied in plastic-based consumer products, including packaging materials, children's toys, medical devices, clothing, and personal care products, such as cosmetics. In addition, the majority of phthalates have a low melting point (below -25°C) and high boiling point (range from 230°C to 486°C), which contributes to their widespread application as heat transfer fluids and carriers (Abdel daiem et al., 2012). The worldwide production of phthalates grew from 1.8 to 4.3 million tons between 1970 and 2006, and increased to more than 8 million tons in 2011 (Habert et al., 2010; Peijnenburg & Struijs, 2006; Schreiber et al., n.d.). To date, approximately 6-8 million tons of phthalates are consumed annually (Net et al., 2015).

Although phthalates are of great economic and commercial interest, there is mounting concern about the association of phthalates with several adverse health problems, such as disruption of reproductive and immune systems (Berger et al., 2018; Watkins et al., 2017). Phthalates are classified as Endocrine Disruptor Chemicals (EDCs), a group of substances that impair the human endocrine system (Katsikantami et al., 2016). EDCs can interfere with endogenous hormone signaling pathways, inducing alterations in the reproductive system, hormone-dependent cancers, and metabolic disorders, such as obesity and T2D (Swedenborg et al., 2009). The alarmingly adverse impact of phthalates on public health has attracted considerable attention and consequently led to restrictions on the usage of phthalates in certain products. As a result, some types of phthalates, such as di-2-ethylhexylphthalate (DEHP) and dibutyl phthalate (DBP), are limited or forbidden in some consumer products (Planelló et al., 2011; EU/2005/84/EC, 2005).

Since phthalates are not chemically bound to plastics, they can gradually leach and migrate into the air, food, water, and other materials (Bertelsen et al., 2013; Heudorf et al., 2007), leading to contamination of the environment. Furthermore, the degradation of phthalates is very slow under natural conditions (Gao & Wen, 2016). Therefore, phthalates can persist in the environment and accumulate along the food chain. Indeed, phthalates have been detected in the air, food, and water, thereby exposing animals and humans. Phthalates can also enter the human body via several routes, i.e., inhalation, ingestion, and dermal contact. Based on exposure assessment by modeling ambient exposure data, the primary source of human exposure is through food and water intake, but low-molecular-weight phthalates, such as diethyl phthalate (DEP) and DBP, may also be dermally absorbed (Calafat & McKee, 2006). Humans can also get exposed to phthalates from medical devices during medical procedures (e.g., hemodialysis) and pharmaceuticals where phthalates are used as excipients (Kelley et al., 2012). In addition, it has been reported that phthalate metabolites can be detected in human urine, blood, breast milk, and sweat samples (Genuis et al., 2012; Lottrup et al., 2006).

Insulin resistance of skeletal muscle in T2D

Insulin resistance refers to an impaired ability of tissues, such as skeletal muscles, adipose tissues, and the liver, to take up glucose under insulin stimulation. Skeletal muscle is the primary site of insulin-stimulated glucose uptake, accounting for approximately 60% of glucose disposal in the postprandial phase (DeFronzo et al., 1981). Since skeletal muscles play an essential role in insulin-mediated glucose disposal, insulin resistance in skeletal muscle is considered a critical component of whole-body insulin resistance in diseases, including T2D

(Turcotte & Fisher, 2008). Both lean and obese T2D patients have markedly decreased insulin sensitivity in skeletal muscles (Butterfield & Whichelow, 1965). Moreover, insulin resistance in skeletal muscle is viewed as one of the initiating factors of T2D, which develops decades before insufficient insulin secretion and hyperglycemia (Defronzo & Tripathy, 2009; Lauro et al., 1998).

In skeletal muscle cells, insulin promotes the influx of glucose by triggering a cascade of phosphorylation and dephosphorylation reactions (Sylow et al., 2021) (Fig. 1). When insulin binds to insulin receptors (IRs) at the cell surface plasma membrane, it activates the phosphorylation of tyrosine residues on IRs (Karlsson & Zierath, 2007). Subsequently, phosphorylated IRs recruit insulin receptor substrate (IRS) proteins. In skeletal muscle cells, IRS-1 has been demonstrated to regulate GLUT4 translocation (Huang et al., 2005), and phosphorylated IRS-1 leads to phosphatidylinositol 3-kinase (PI3K) activation. Activated PI3K phosphorylates phosphatidylinositol 4, 5-bisphosphate (PIP_2) to generate phosphatidylinositol 3, 4, 5-triphosphate (PIP_3), which activates protein kinase B (Akt), facilitating the translocation of GLUT4 to the plasma membrane. While cycling between intracellular space and the plasma membrane, most GLUT4 resides in GLUT4 storage vesicles (GSVs) in the basal state (Jaldin-Fincati et al., 2018; Knudsen et al., 2019). Activation of the insulin signaling in skeletal muscle stimulates GLUT4 to move from the intracellular pool to the cell membrane. In diseases characterized by insulin resistance, such as T2D, glucose transport activity is severely impaired. Muscle biopsies from diabetic rats and non-insulin-dependent diabetes mellitus (NIDDM) patients show a normal level of GLUT4 mRNA and protein, thus demonstrating that insulin resistance in skeletal muscle is not caused by GLUT4 depletion but rather impaired GLUT4 function or translocation (Eriksson et al., 1992; Garvey et al., 1992, p. 4; Kahn et al., 1991).

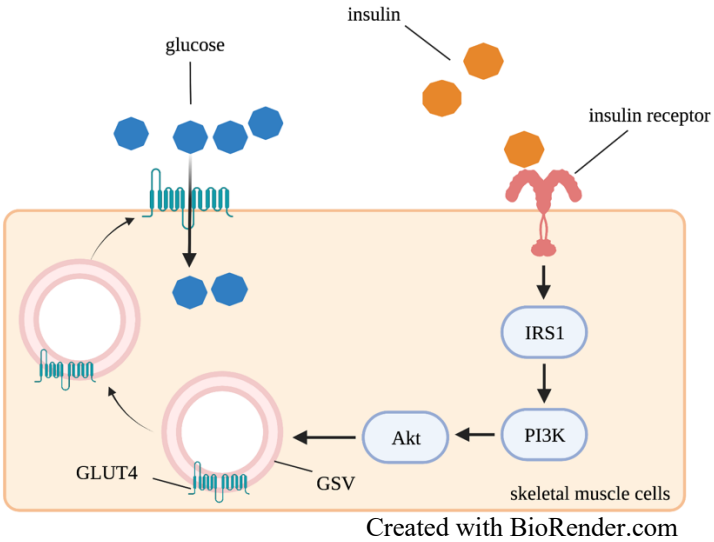

Fig. 1. Insulin-mediated glucose uptake in skeletal muscle cells. Insulin binds to insulin receptors (IRs) on the surface of skeletal muscle cells, triggering the phosphorylation of IRs and consequently the recruitment and activation of insulin receptor substrate (IRS) proteins. IRS-1 leads to phosphatidylinositol 3-kinase (PI3K) activation, which will further activate protein kinase B (Akt), motivating GLUT4 containing vesicles to move to the plasma membrane and GLUT4 to insert into the plasma membrane.

Phthalate exposure is associated with T2D

Long-term exposure to phthalates and their metabolites has been associated with detrimental health problems, including obesity and T2D. There is emerging evidence that phthalates play a role in the development of T2D. Epidemiological studies have associated exposure to phthalates with increased risk for T2D (Kim & Park, 2014; Lind et al., 2012; Radke et al., 2019; Sun et

al., 2014). Insulin resistance is a characteristic feature of T2D. Phthalate exposure has been associated with insulin resistance in both adults and adolescents. A cross-sectional study of 766 children aged 12-19 shows that urinary DEHP concentrations are associated with increased insulin resistance (Trasande et al., 2013). The 1999-2002 National Health and Nutrition Examination Survey (NHANES) from the U.S. has identified strong correlations of high-molecular-weight phthalate metabolites with abdominal obesity and insulin resistance in adult men (Stahlhut et al., 2007).

Several recent studies have attempted to obtain a better understanding of the molecular mechanisms linking phthalate exposure with T2D. DEHP has been shown to adversely affect IR and GLUT4 at transcriptional and translational levels in L6 myotubes (Rajesh & Balasubramanian, 2014). At the molecular level, DBP has been shown to aggravate T2D by disrupting the insulin-mediated PI3K/AKT signaling pathway and decreasing the glucose transporter type 2 (GLUT2) level in the pancreas (Deng et al., 2018). Maternal exposure to DBP aggravates gestational diabetes mellitus (GDM) in rats and impairs islet β cells' cell viability via suppression of Forkhead box protein M1 (FoxM1)-mediated pathway (M. Chen et al., 2020, p.).

However, our knowledge of the effects of DBP on insulin sensitivity of skeletal muscles is still lacking. Furthermore, unlike GLUT2 being the primary glucose transporter in β-cells, GLUT4 is the predominant isotype expressed on skeletal muscle cells, and impaired GLUT4 expression or translocation is a characteristic feature of skeletal muscle insulin resistance in T2D (Zierath et al., 2000). Thus, it could be of great interest to study if DBP affects insulin sensitivity and GLUT4 membrane translocation in skeletal muscles.

Aims

This study aims to investigate the effects of DBP exposure on insulin sensitivity in skeletal muscle cells. Specific aims include 1) investigating the effects of DBP exposure on insulin-stimulated glucose uptake in skeletal muscle cells, 2) studying whether DBP exposure affects cell viability in skeletal muscle cells, and 3) examining the GLUT4 membrane translocation in skeletal muscle cells treated with DBP.

Materials and methods

Materials

Mouse C2C12 myoblast cell line was purchased from Leibniz Institute DSMZ (Braunschweig, Germany). Fetal bovine serum (FBS), horse serum (HS), Deulbecco's modified eagle medium (DMEM), 100 mm tissue culture dishes, glucose-free DMEM, penicillin-streptomycin-glutamine (100×) (P/S), phosphate-buffered saline (PBS), trypsin (2.5%), dimethyl sulfoxide (DMSO), PrestoBlue™ HS cell viability reagent, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), PureLink™ DNase Set, PureLink™ RNA mini kit, high-capacity RNA to cDNA kit, 96-/6-well tissue culture plates, Nunc™ Lab-Tek™ II Chamber Slide™ System, GLUT4 rabbit polyclonal antibody, Cy3 goat anti-rabbit IgG (H+L), NucBlue™ fixed cell ReadyProbes™ reagent (DAPI), Tween-20, 4% formaldehyde were purchased from Thermo Fisher Scientific (Stockholm, Sweden). 70% ethanol was purchased from Solveco AB (Stockholm, Sweden). Insulin, 100% acetic acid, DBP, bovine serum albumin (BSA), Triton x-100, and 1% β-Mercaptoethanol (2-ME) were purchased from Sigma-Aldrich (Darmstadt, Germany). iTaq™ universal SYBR® green supermix was purchased from Bio-Rad (Stockholm, Sweden).

Cell culture and differentiation

Mouse myoblast C2C12 cell line has been used widely in vitro as a model for understanding insulin resistance in T2D due to its expression of GLUT4 and other features that resemble human skeletal muscle cells (Wong et al., 2020). Mouse C2C12 myoblasts were maintained as a monolayer in 100 mm tissue culture dishes containing 10 mL of growth medium (DMEM containing 10% (v/v) FBS, 1% (v/v) P/S) at 37℃ under a humidified condition of 95% air and 5% CO_2. C2C12 myoblasts were trypsinized using diluted trypsin (0.25%) and split into 1:5 every three days. Only C2C12 myoblasts in earlier passages (from 2 up to 15 times) were used in this study.

C2C12 myoblasts were differentiated using a method previously described (Liu et al., 2016, p. 1). Briefly, when cells reached 90-100% confluence, the growth medium was changed to the differentiation medium (DMEM containing 2% (v/v) HS and 1% (v/v) P/S). During differentiation, the medium was changed every other day. Cells in cell culture with at least 95% conversion into the myogenic morphology were considered myotubes (He et al., 2017). After five days of differentiation, C2C12 myotubes formed.

DBP preparation and treatment

DBP was dissolved in DMSO (cat. no.20688, Thermo Scientific) to the final concentrations of 0.1 nM, 1 nM, 10 nM, 100 nM and 1 μM. 0.1% DMSO was used as the control. DBP or 0.1% DMSO was added to the cell culture from the beginning of C2C12 myoblast differentiation to 5 days after C2C12 myotube formation. During the 10-day treatment, DBP and 0.1% DMSO were renewed every other day along with the differentiation medium. DBP exposure strategy was described in Fig. 2.

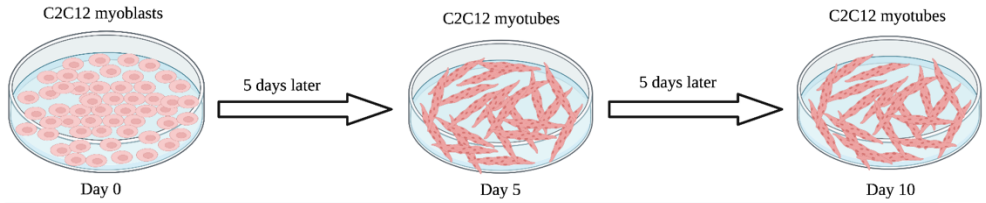

Fig. 2. Schematic diagram of DBP exposure strategy. The differentiation process started when cells reached 90-100% confluence. Different DBP concentrations (0.1 nM, 1 nM, 10 nM, 100 nM or 1 μM) or 0.1% DMSO control were added to the cell culture at the beginning of myoblast differentiation. Cells collected on Day 5 were considered myotubes. DBP or 0.1% DMSO exposure continued for 5 days after myotube formation. Cells collected on Day 10 were used in the following experiments.

Total RNA extraction

C2C12 myoblasts were dissociated from the 100 mm culture dishes using 2 mL of 0.25% trypsin. After trypsinization and centrifugation, cells pellets were resuspended in growth medium and inoculated in 6-well tissue culture plates with 50,000 cells/mL seeding density. C2C12 myoblast differentiation was the same as in the "Cell culture and differentiation" section. Total RNA extraction was performed on Day 0 and Day 5 using PureLink™ RNA mini kit (cat. no.12183025, Invitrogen) according to the manufacturer's instructions. Lysis buffer, Spin Cartridge, Wash Buffer I, Collection Tube, RNase-free water, and Wash buffer II were provided within the kit. Briefly, cells were lysed by Lysis Buffer with 1% 2-ME (cat. no.60242, Sigma-Aldrich) added. One volume of 70% ethanol (cat. no.12345, Solveco AB) was added to each volume of cell homogenate, and the mixture was vortexed to mix it thoroughly. Cell homogenate was transferred to the Spin Cartridge and centrifuged at 12,000 x g for 15 seconds at room temperature (RT). After centrifugation, the flow-through was discarded, and Wash Buffer I was added to the Spin Cartridge, which was then centrifugated at 12,000 x g for 15 seconds at RT. The membrane of the Spin Cartridge was incubated with DNase (cat. no.12185010, Invitrogen) for 15 minutes and then washed with Wash Buffer I. The Spin Cartridge was placed into a new Collection Tube and washed with Wash buffer II twice. The membrane bound with RNA was dried by centrifuging Collection Tube at 12,000 x g for 1 minute at RT. RNA was eluted by incubating the membrane of Spin Cartridge with 30 μL of RNase-free water followed by centrifugation at 12,000 x g for 2 minutes at RT. RNA concentration was measured by Multiskan GO spectrophotometer (Thermo Scientific, Sweden). RNA samples were stored at -80°C until cDNA synthesis.

cDNA synthesis

cDNA was synthesized using high-capacity RNA to cDNA kit (cat. no. 4387406, Applied Biosystems) according to the manufacturers' instructions. For each sample, 1 μg of RNA was used in every reverse transcription (RT) reaction. For each RT reaction, 10 μL of RT Buffer Mix and 1 μL of Enzyme mix were added. RNase-free water was added to each RT reaction to quantify the total volume of 20 μL. RT reaction was performed using LifeECO thermal cycler (Bioer Technology, Sweden): incubation at 37°C for 60 minutes, heating to 95°C for 5 minutes, and the samples were held at 4°C. cDNA samples were stored at -20°C.

Quantitative PCR (qPCR)

The mRNA levels of the housekeeping gene (β-actin) and genes of interest (SERCA1 and Cavolin3) were measured using qPCR. qPCR was performed in CFX Connect Real-Time Detection System (Bio-Rad, Sweden). Amplification was achieved by iTaq™ universal SYBR® green supermix (cat. no.1725121, Bio-Rad). Each sample had three technical replicates; for each primer pair, one negative control with RNase-free water was included on each plate.

Primer sequences were designed according to published lectures (Benhaddou et al., 2012; Chai et al., 2010; Tang et al., 2018), and primer sequences were listed in Table 1. The standard temperature profile included preincubation at 95℃ for 1 minute, followed by 45 cycles of 95℃ for 10 seconds, 60℃ for 30 seconds, and 72℃ for 30 seconds. The Delta-Delta Ct method was used to determine the relative mRNA expression using β-actin as reference.

Table 1. Primer sequences used for qPCR.

Gene	Forward primer	Reverse primer
β-actin	*ACCAGTTCGCCATGGATGAC*	*TGCCGGAGCCGTTGTC*
SERCA1	*GCGAGGTGGTCTGTATCTTCTTG*	*TGTCCAGGTCAGGTGGCTTG*
Caveolin 3	*ACATGTGCCAAGGTCACTCA*	*CCTTGGAGTCCCTGGAATTT*

2-NBDG uptake assay

2-NBDG (cat. no.N13195, Invitrogen) was used to assess the insulin-stimulated glucose uptake in C2C12 myotubes. 50,000 cells/mL were seeded in 6 well-plates. The differentiation method and DBP treatment were described in the "Cell culture and differentiation" and "DBP preparation and treatment" sessions, respectively. Afterward, cells were incubated in glucose-free DMEM medium for 2 hours before insulin stimulation. Post starvation, cells were trypsinized and re-suspended in PBS containing 100 nM insulin (cat. no. I6634, Sigma-Aldrich) and 50 μM 2-NBDG for 30 minutes at 37℃ in the dark. Reaction was stopped by washing with cold PBS twice. The mean fluorescence intensity (MFI) of cells containing 2-NBDG was measured using the CytoFlex S flow cytometer (Beckman Coulter).

Cell viability assay

C2C12 myoblasts were trypsinized and resuspended in growth medium, plated in 96-well tissue culture plates with 50,000 cells/mL seeding density, and allowed to attach for 24 hours in a 5% CO_2 humidified incubator at 37℃. The differentiation method and DBP exposure were the same as in the "Cell culture and differentiation" and "DBP preparation and treatment" sessions, respectively. After DBP or 0.1% DMSO exposure, culture medium was replaced with 90μL of differentiation medium, and 10μL of PrestoBlue™ HS cell viability reagent (cat. no.P50200, Invitrogen) was added to each well. Plates were wrapped with foil to avoid direct light and incubated for 2 hours at 37℃ in a cell culture incubator. The absorbance of the reagent at 570nm and 600nm were then measured by Multiskan GO spectrophotometer (Thermo Scientific, Sweden). 570nm values were normalized to 600nm.

Immunofluorescence staining of GLUT4 in C2C12 myotubes

GLUT4 membrane translocation was detected by immunofluorescence staining. C2C12 myoblasts were seeded in Nunc™ Lab-Tek™ II Chamber Slide (cat. no.154534, Thermo Scientific) with 50,000 cells/mL seeding density. C2C12 myotube differentiation and DBP treatment were described in the "Cell culture and differentiation" and "DBP preparation and treatment" sections. The cells were incubated in glucose-free DMEM medium for 2 hours and then treated with 100 nM insulin for 30 minutes. After being fixed in 4% formaldehyde (cat. no.FB0002, Invitrogen) for 20 minutes at RT, cells were permeabilized using 0.1% Triton X-100 (cat. no.9036195, Sigma-Aldrich) for 10 minutes at RT. After fixation and permeabilization, cells were washed with PBS twice and incubated with 2% BSA (cat. no.9048468, Sigma-Aldrich) for 1 hour at RT. The cells were washed with PBS twice and incubated with GLUT4 rabbit antibody (1:100) (cat. no.PA523052, Invitrogen) at 4℃ overnight. The cells were washed and incubated in PBST (0.1% Tween-20 diluted in PBS) for 10 minutes at RT three times, followed by incubation with Cy3 goat anti-rabbit IgG (H+L) (1:500) (cat. no.31210, Invitrogen) for 1 hour at RT. Cells were rinsed with PBS and stained with DAPI (cat. no.R37606, Invitrogen)

for 15 minutes at RT. Images were taken using LSM 700 confocal microscope (ZEISS, Germany).

Statistical analyses

Mean and standard errors from all replicates of each experiment were calculated. Statistical analyses were performed using GraphPad Prism 9. Unpaired Student's *t*-test was used for two independent group comparisons, and one-way ANOVA was used for multiple comparisons.

Results

C2C12 myotube differentiation was verified by morphological change and RT-qPCR

After five days of differentiation, C2C12 myoblasts fused into myotubes (Fig. 3A and B). The differentiation of C2C12 myotubes was further confirmed by measuring relative mRNA expression levels of SERCA1 and Caveolin 3, which are expressed in myotubes but not myoblasts (Harrer et al., 1995; Song et al., 1996). The relative mRNA expression levels of SERCA1 and Cavolin3 significantly increased in C2C12 myotubes than C2C12 myoblasts ($p < 0.05$) (Fig. 3C).

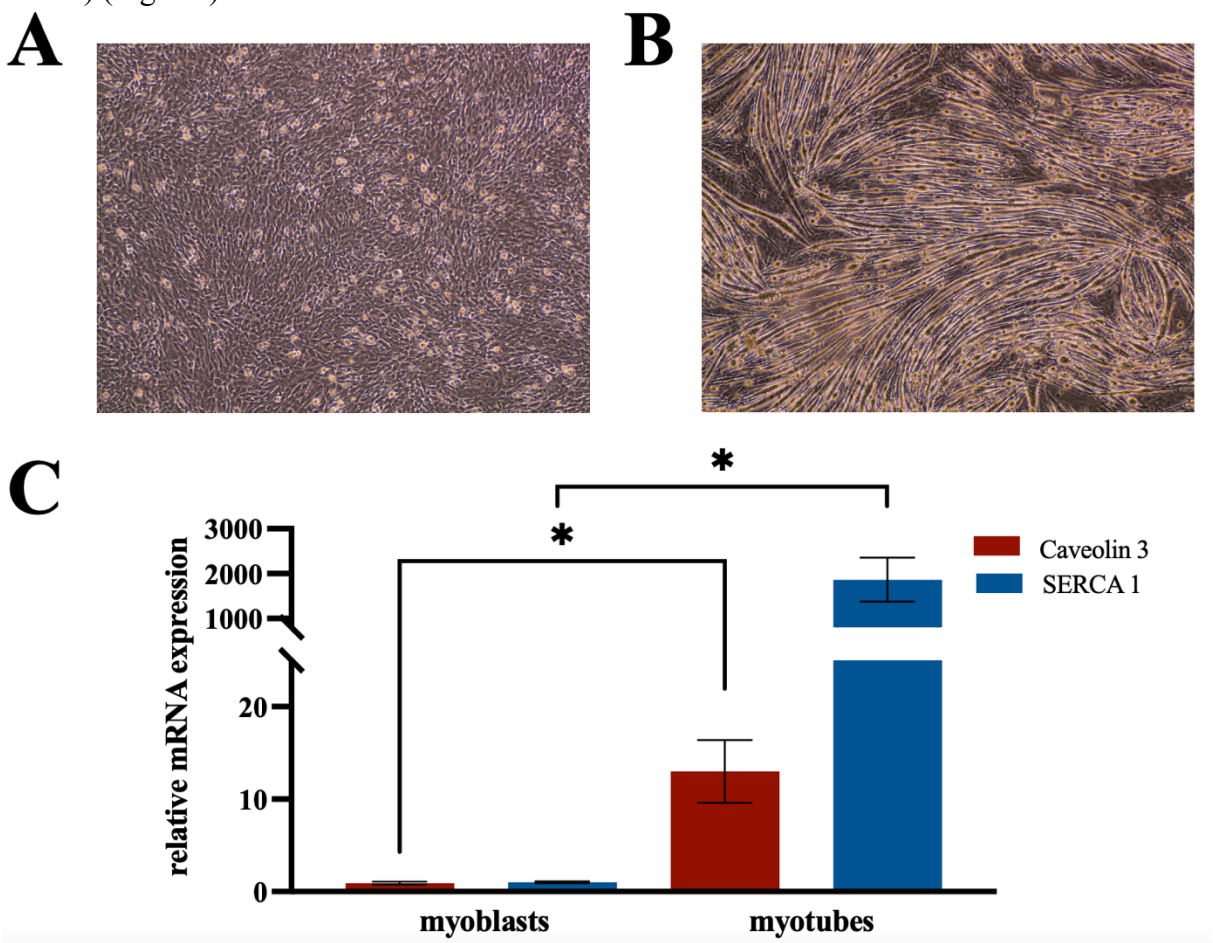

Fig. 3. C2C12 myotubes differed from myoblasts in morphology and genes at the transcriptional level. After five days of differentiation, C2C12 myoblasts (A) fused into C2C12 myotubes (B). Relative expression levels of SERCA1 and Caveolin 3 were compared between C2C12 myoblasts and C2C12 myotubes (C). Values represented mean ± SD from three independent experiments. Error bars indicate SEM. (*$p < 0.05$; Student's *t*-test).

DBP exposure (10nM, 100nM, and 1μM) reduced insulin-stimulated 2-NBDG uptake in C2C12 myotubes

To find whether DBP exposure affects insulin sensitivity, we assessed 2-NBDG uptake under insulin stimulation in C2C12 myotubes treated with 0.1 nM, 1 nM, 10nM, 100nM, and 1μM DBP. Our results showed that DBP-induced reduction of insulin-stimulated 2-NBDG uptake in C2C12 myotubes was dose-dependent (Fig. 4). Exposure to DBP at doses of 10 nM, 100 nM and 1 μM significantly decreased insulin-mediated 2-NBDG uptake in C2C12 myotubes.

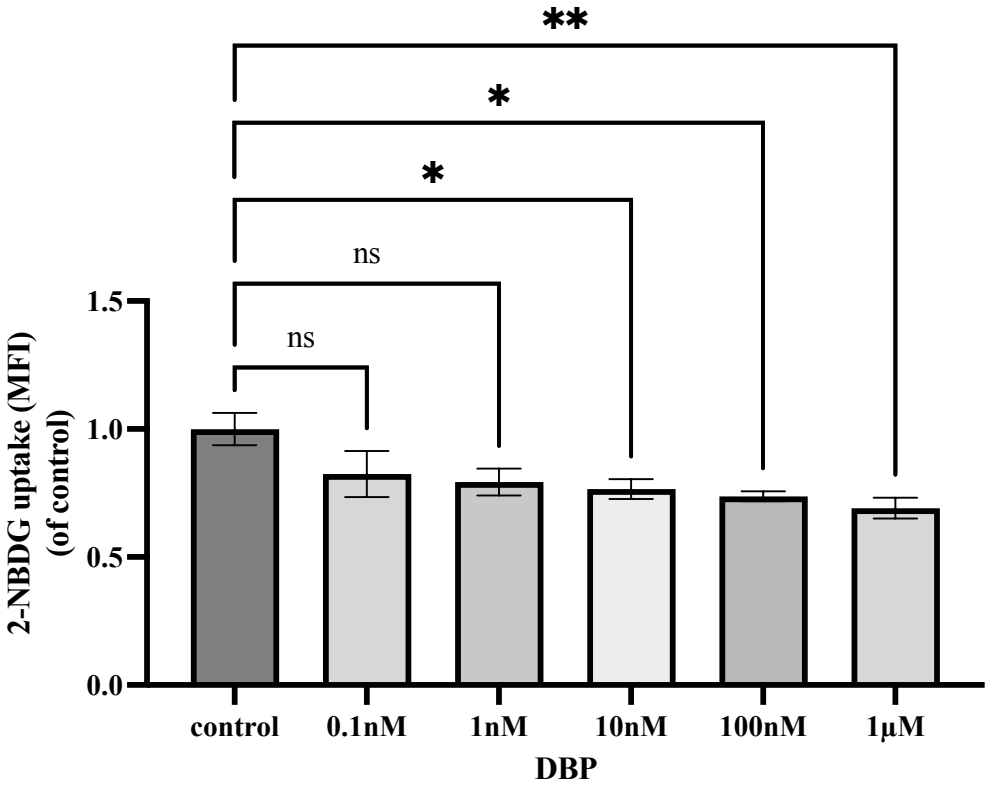

Fig. 4. DBP exposure (10nM, 100nM, and 1μM) impaired insulin-mediated 2-NBDG uptake in C2C12 myotubes. Results were shown as mean ± SD from three independent experiments. Error bars indicate SEM. (ns > 0.05, *p < 0.05, **p < 0.01; ordinary one-way ANOVA with Dunnett's multiple comparisons test).

DBP exposure did not affect C2C12 myotubes' cell viability

Phthalates, such as diethyl phthalate (DEP) and DBP, have been reported to damage cell viability (Yin et al., 2018). Therefore, to evaluate whether the decreased insulin-stimulated 2-NBDG uptake was due to the cytotoxicity of DBP, we analyzed the cell viability of DBP-treated C2C12 myotubes. Results showed that DBP exposure (0.1 nM, 1 nM, 10nM, 100nM, and 1μM) did not affect the cell viability in C2C12 myotubes (Fig. 5). Taken together with the insulin-stimulated 2-NBDG uptake result, it suggested that DBP (10nM, 100nM, and 1μM) impairs insulin sensitivity in C2C12 myotubes.

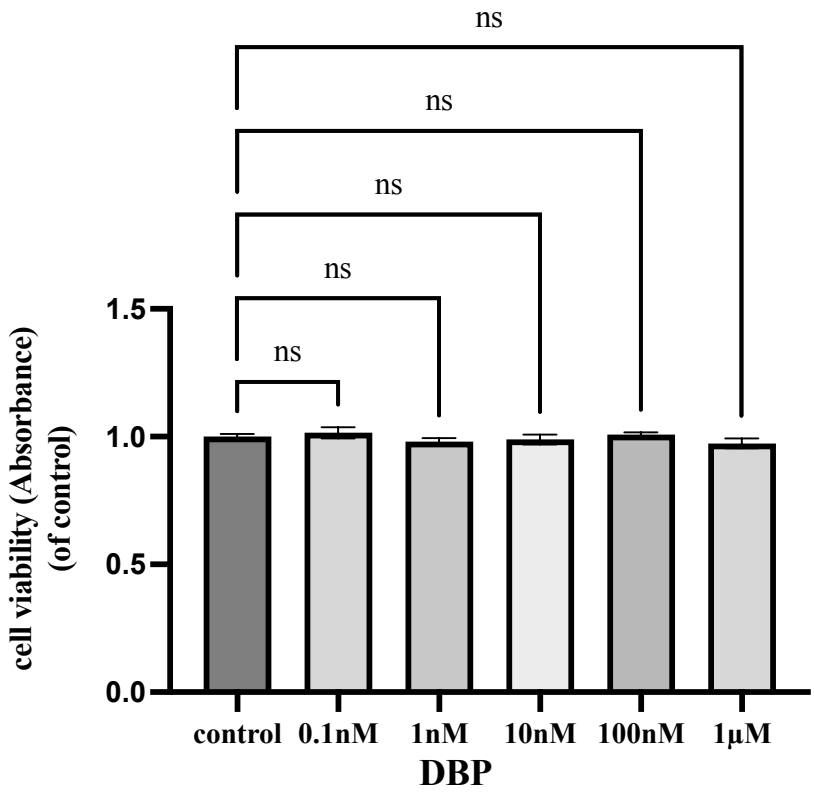

Fig. 5. DBP exposure (0.1 nM, 1 nM, 10nM, 100nM, or 1μM) does not reduce the cell viability of C2C12 myotubes. Results were shown as mean ± SD from six independent experiments. Error bars indicate SEM. (ns > 0.05; ordinary one-way ANOVA with Dunnett's multiple comparisons test).

DBP exposure (10 nM and 100 nM) reduced GLUT4 membrane translocation in C2C12 myotubes

Insulin-mediated glucose uptake is primarily mediated by GLUT4. GLUT4 vesicles are stored in the cytoplasm at basal condition and can be motivated to insert into the cell membrane in response to insulin. Therefore, impaired GLUT4 translocation might contribute to the reduced insulin-stimulated glucose uptake in DBP-treated cells. To investigate if DBP exposure (10nM and 100nM) led to reduced GLUT4 translocation on the cell membrane, we further evaluated the GLUT4 distribution in C2C12 myotubes using immunofluorescence staining. As shown in Fig. 6, there was more GLUT4 (red) located on the cell membrane than in the intracellular compartment in the control sample, whereas there was no such evident difference observed in 10 nM and 100 nM DBP samples.

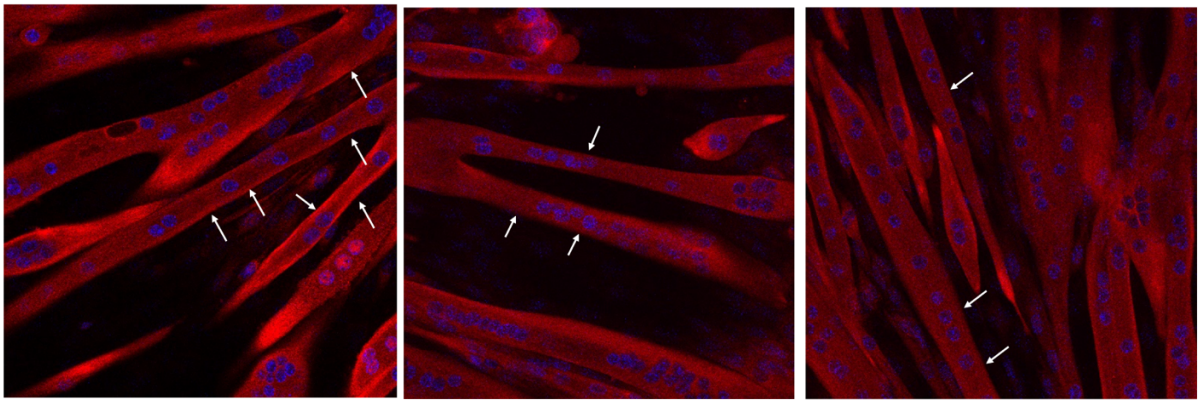

Fig. 6. Effects of GLUT4 distribution in DBP (10 nM and 100 nM)-treated C2C12 myotubes. The red color indicated GLUT4 bound with Cy3 labeled secondary antibody. The blue color indicated that the nucleus was stained with DAPI. The white arrows indicated where the red fluorescence signal appeared relatively stronger on the cell membrane. Compared to the control, it seemed that in DBP-treated cells, the red fluorescence on the membrane was relatively weaker.

Discussion

Phthalates are endocrine disruptor chemicals (EDCs) that industries use for the manufacture of various daily products, including food packaging and personal care products. In epidemiological studies, there are indications that exposure to phthalates increases the risk for T2D; nonetheless, the molecular mechanisms linking these chemicals and T2D are not clearly understood. Six different types of phthalates can be found in consumer products: DEHP, DBP, diisononyl phthalate (DINP), diisodecyl phthalate (DIDP), dioctyl phthalate (DNOP), and benzyl butyl phthalate (BBP) (Buñay et al., 2017). Low molecular weight phthalates with short side chain such as DBP are more water-soluble than the phthalates with long alkyl group such as DEHP (Lyche, et al., 2011), suggesting higher propensity of DBP contaminating drinking water and food. Moreover, DBP has been used as solvents in numerous cosmetics, increasing its risk of entering human body through dermal contact (Lyche, et al., 2011). Humans are chronically exposed to DBP throughout their lives; hence, the metabolic consequences of DBP are of major concern but not elucidated so far. These questions led us to investigate the effects of DBP on insulin sensitivity in C2C12 cells.

An important concept in the study of environmental endocrinology is low dose exposures (LDEs) of EDCs. LDEs has been defined by the U.S Environmental Protection Agency (EPA) as any biological changes occurring in the typical human exposure range or at doses lower than those used in traditional toxicology tests (Melnick et al., 2002). Studies on LDEs have revealed that EDCs can have adverse effects at low doses but not high doses, which challenged the traditional "the dose makes the poison" concept in the field of toxicology (Vandenberg et al., 2012). For example, prenatal low-dose exposure to BPA (1 μg/mL in drinking water) induced obesity in female offspring, while adipose tissue weight of female offspring was not significantly increased with high-dose of BPA (10 μg/mL in drinking water) (Miyawaki et al., 2007). According to published data on estimated phthalate exposure levels, the median and the interquartile range of daily exposures for DBP are 0.84 and 1.77 μg/kg/day, respectively (Marsee et al., 2006; Swan et al., 2005). For example, for a person with 60 kg body weight, the daily exposure of DBP is 213 nM per day. Therefore, based on these prior studies, we chose a concentration gradient from 0.1 nM to 1 μM of DBP in this study.

Insulin sensitivity represents the ability of insulin target tissues (e.g., adipocytes, skeletal muscle cells, and neurons) to take up glucose in response to insulin. Impaired insulin sensitivity, or insulin resistance in skeletal muscle is one of the primary factors underlying T2D (Turcotte & Fisher, 2008). Studies have demonstrated that phthalates and their metabolites can interact with peroxisome proliferator-activated receptors (PPARs) activity (Feige et al., 2007, 2010), which is worth noting because PPARs are involved in lipid metabolism and insulin sensitivity (Ye et al., 2001, p.). Although there is evidence that DBP can influence all three PPARs isotypes (PPAR-α, -β/δ, -γ) in cellular models (Lapinskas et al., 2005), the biological effects of DBP have remained elusive so far. In the present study, we used 2-NBDG uptake assay to quantitatively measure insulin sensitivity in C2C12 cells and observed that insulin-stimulated 2-NBDG uptake in C2C12 cells treated with 10 nM, 100 nM, and 1 μM DBP significantly decreased, suggesting DBP exposure impairs insulin sensitivity in skeletal muscle cells. Additionally, our results showed that the DBP-induced decrease of insulin-mediated glucose uptake in C2C12 cells was dose-dependent.

To our knowledge, there has been no published data on how DBP exposure affects insulin sensitivity in cultured skeletal muscle cells. However, there are some studies investigating the effects of other members of phthalates, such as DEHP. Gestational exposure to DEHP leads to abnormal β cell function, reduced insulin secretion, and impaired glucose tolerance in rat

offspring (Lin et al., 2011). A previous report has demonstrated that acute exposure (24 hours) to DEHP (100 μM) and its metabolite MEHP (50 or 100 μM) resulted in a significant decrease in insulin-stimulated glucose uptake in L6 myotubes, the rat skeletal muscle model (Viswanathan et al., 2017). Compared to these studies, in our study, the dose of DBP treatment is around estimated human daily exposure level, and the exposure time is longer, which is more representative of human's daily exposure to circulating levels of phthalates in the environment. We are exposed to phthalates during our whole life, including intrauterine development. In the present study, DBP exposure also persisted throughout myoblast differentiation and myotube stage. Therefore, our study provides a new insight to the molecular mechanism of phthalates' association with diseases characterized by insulin resistance, such as obesity and T2D.

Since, in our study, DBP exposure started from the beginning of myoblast differentiation, it is possible that DBP exposure caused disrupted myogenesis and, consequently, impaired insulin sensitivity in C2C12 cells. Indeed, it has been reported that gestational exposure to phthalates leads to reduced body weight at birth and restricted growth during development in newborn rodents (S.-Q. Chen et al., 2010; Tanida et al., 2009). In vitro studies have also shown that phthalate exposure decreases human endometrial mesenchymal stem cells (EN-MSCs) myogenetic differentiation (H.-S. Chen et al., 2017) and C2C12 myoblast differentiation (S.-S. Chen et al., 2013). However, in our study, we did not observe an evident decrease in the elongation of multinucleated myotubes in C2C12 cells treated with DBP (data not shown). To better understand whether DBP disrupts myogenesis in C2C12 cells, improvements can be made to our study, for example, detecting the expression of the muscle-specific markers, i.e., myosin heavy chain (MyHC) and genes regulating myogenic differentiation, i.e., MyoD and myogenin.

Another possible explanation for DBP-induced insulin resistance in C2C12 cells could be the cytotoxicity effect of DBP. The cytotoxic effect of phthalates on cell viability has been reported. A previous study demonstrated that 10 nM DEHP exposure resulted in lower viability and higher apoptosis in human granulosa cells (GCs) and steroidogenic human granulosa-like tumor cell line (KGN cells) (Jin et al., 2019). Another study revealed that exposure to DEHP (50 μM, 100 μM, 200 μM, 400 μM, and 800 μM) for 48 hours and 72 hours caused significant inhibition of cell viability in L6 myotubes; whereas 25 μM DEHP treatment for 72 hours did not affect cell viability (Rajesh & Balasubramanian, 2014). Therefore, to examine whether the inhibition of insulin-stimulated glucose uptake in DBP-exposed cells was caused by the cytotoxicity of DBP, we went on to assess the cell viability in DBP-treated C2C12 cells. Our results revealed that DBP in the chosen concentration gradient did not reduce cell viability, indicating that the impaired insulin-mediated 2-NBDG uptake in DBP-treated C2C12 cells might be due to the disruption of insulin-mediated glucose transportation pathway, such as insulin signaling and GLUT4 membrane translocation.

Activation of the insulin signaling in skeletal muscle stimulates GLUT4 to move from the intracellular pool to the plasma membrane. In diseases characterized by insulin resistance, such as T2D, GLUT4 translocation is severely impaired. To decipher how DBP causes reduced insulin sensitivity in C2C12 cells, we further analyzed the GLUT4 translocation in C2C12 cells treated with 10 nM or 100 nM DBP. Our results showed that DBP-treated C212 cells exhibited less GLUT4 on the cell membrane, suggesting diminished GLUT4 translocation might play a role in insulin resistance in C2C12 cells treated with 10 nM or 100 nM DBP. However, this finding needs to be taken with great caution. Since the cytosolic and cell membrane GLUT4 protein level are not quantitively measured in the present study, there is the possibility that the decreased GLUT4 expression on the cell membrane in DBP-treated C2C12 cells is caused by

decrease in GLUT4 production. To better understand the effects of DBP on GLUT4 in C2C12 cells, improvements can be done to our study, for instance, transfecting C2C12 cells with HA-GLUT4-GFP construct to study the ratio between intracellular and plasma membrane translocated GLUT4 (Heckmann et al., 2022; Lizunov et al., 2012).

One of the continuations of this study is to investigate pathways and molecules involved in DBP-induced insulin resistance in C2C12 cells. Future studies on how DBP affects insulin signaling pathways, proteins responsible for moving GLUT4-containing vesicles to the plasma membrane, and glucose metabolism will help us better understand DBP-induced insulin resistance in skeletal muscle cells. Moreover, when DBP enters our body, it is decomposed into metabolites, such as mono-(3-carboxypropyl) phthalate (MCPP). Whether these metabolites yield the same effect as DBP on skeletal muscle cells is still unknown. Thus, in vitro studies, including DBP metabolites, is also of great interest. Eventually, more research needs to be performed on how DBP affects living animals.

To conclude, DBP exposure (10 nM, 100 nM, and 1 μM) impairs insulin sensitivity in C2C12 cells, and it is not due to the cytotoxicity effect of DBP. DBP (10nM and 100nM)-exposed C2C12 cells might exhibit decreased GLUT4 membrane translocation.

Acknowledgments

I would like to thank my supervisor Wen for her expertise, patience, enthusiasm, and passion for science. Thank you for being optimistic and supportive when I was down, for being critical and cautious when I was over the roof, and for being a good friend to whom I can talk. It has been a joy to work with you. I would like to extend my gratitude to postdoc Chengxi. Thank you for giving me invaluable advice on immunofluorescence staining protocol and for improving my experimental skills.

I would like to thank all members in the cell lab and the fly lab, including Ines, Tim, Patricia, Tri, Anna, Hasnat, and Nishant. Thank you for your company and encouragement. I have learned a lot from you. And I wish you all a happy and bright future.

Finally, I would like to thank my mom and dad, who have always been supportive.

References

Abdel daiem, M. M., Rivera-Utrilla, J., Ocampo-Pérez, R., Méndez-Díaz, J. D., & Sánchez-Polo, M. (2012). Environmental impact of phthalic acid esters and their removal from water and sediments by different technologies – A review. *Journal of Environmental Management*, *109*, 164–178. https://doi.org/10.1016/j.jenvman.2012.05.014

Benhaddou, A., Keime, C., Ye, T., Morlon, A., Michel, I., Jost, B., Mengus, G., & Davidson, I. (2012). Transcription factor TEAD4 regulates expression of Myogenin and the unfolded protein response genes during C2C12 cell differentiation. *Cell Death & Differentiation*, *19*(2), 220–231. https://doi.org/10.1038/cdd.2011.87

Berger, K., Eskenazi, B., Kogut, K., Parra, K., Lustig, R. H., Greenspan, L. C., Holland, N., Calafat, A. M., Ye, X., & Harley, K. G. (2018). Association of Prenatal Urinary Concentrations of Phthalates and Bisphenol A and Pubertal Timing in Boys and Girls. *Environmental Health Perspectives*, *126*(9), 097004. https://doi.org/10.1289/EHP3424

Bertelsen, R. J., Carlsen, K. C. L., Calafat, A. M., Hoppin, J. A., Håland, G., Mowinckel, P., Carlsen, K.-H., & Løvik, M. (2013). Urinary Biomarkers for Phthalates Associated with Asthma in Norwegian Children. *Environmental Health Perspectives*, *121*(2), 251–256. https://doi.org/10.1289/ehp.1205256

Buñay, J., Larriba, E., Moreno, R. D., & del Mazo, J. (2017). Chronic low-dose exposure to a mixture of environmental endocrine disruptors induces microRNAs/isomiRs deregulation in mouse concomitant with intratesticular estradiol reduction. *Scientific Reports*, *7*(1), 3373. https://doi.org/10.1038/s41598-017-02752-7

Butterfield, W. J. H., & Whichelow, M. J. (1965). Peripheral glucose metabolism in control subjects and diabetic patients during glucose, glucose-insulin and insulin sensitivity tests. *Diabetologia*, *1*(1), 43–53. https://doi.org/10.1007/BF01338715

Calafat, A. M., & McKee, R. H. (2006). Integrating Biomonitoring Exposure Data into the Risk Assessment Process: Phthalates [Diethyl Phthalate and Di(2-ethylhexyl) Phthalate] as a Case Study. *Environmental Health Perspectives*, *114*(11), 1783–1789. https://doi.org/10.1289/ehp.9059

Chai, J., Xiong, Q., Zhang, P., Zheng, R., Peng, J., & Jiang, S. (2010). Induction of Ca2+ signal mediated apoptosis and alteration of IP3R1 and SERCA1 expression levels by stress hormone in differentiating C2C12 myoblasts. *General and Comparative Endocrinology*, *166*(2), 241–249. https://doi.org/10.1016/j.ygcen.2009.08.011

Chen, H.-S., Hsu, C.-Y., Chang, Y.-C., Chuang, H.-Y., Long, C.-Y., Hsieh, T.-H., & Tsai, E.-M. (2017). Benzyl butyl phthalate decreases myogenic differentiation of endometrial mesenchymal stem/stromal cells through miR-137-mediated regulation of PITX2. *Scientific Reports*, *7*(1), 186. https://doi.org/10.1038/s41598-017-00286-6

Chen, M., Zhao, S., Guo, W.-H., Zhu, Y.-P., Pan, L., Xie, Z.-W., Sun, W.-L., & Jiang, J.-T. (2020). Maternal exposure to Di-n-butyl phthalate (DBP) aggravate gestational diabetes mellitus via FoxM1 suppression by pSTAT1 signalling. *Ecotoxicology and Environmental Safety*, *205*, 111154. https://doi.org/10.1016/j.ecoenv.2020.111154

Chen, S.-Q., Chen, J.-N., Cai, X.-H., Chen, G.-R., Gao, Y., Ge, R.-S., Wu, H.-S., Lin, Z.-L., & Lin, J. (2010). Perinatal exposure to di-(2-ethylhexyl) phthalate leads to restricted growth and delayed lung maturation in newborn rats. *Journal of Perinatal Medicine*, *38*(5). https://doi.org/10.1515/jpm.2010.083

Chen, S.-S., Hung, H.-T., Chen, T.-J., Hung, H.-S., & Wang, D.-C. (2013). Di-(2-ethylhexyl)-phthalate reduces MyoD and myogenin expression and inhibits myogenic differentiation in C2C12 cells. *The Journal of Toxicological Sciences*, *38*(5), 783–791. https://doi.org/10.2131/jts.38.783

DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., & Felber, J. P. (1981). The Effect of Insulin on the Disposal of Intravenous Glucose: Results from Indirect Calorimetry and Hepatic and Femoral Venous Catheterization. *Diabetes*, *30*(12), 1000–1007. https://doi.org/10.2337/diab.30.12.1000

Defronzo, R. A., & Tripathy, D. (2009). Skeletal Muscle Insulin Resistance Is the Primary Defect in Type 2 Diabetes. *DIABETES CARE*, *32*, 7.

Deng, T., Zhang, Y., Wu, Y., Ma, P., Duan, J., Qin, W., Yang, X., & Chen, M. (2018). Dibutyl phthalate exposure aggravates type 2 diabetes by disrupting the insulin-mediated PI3K/AKT signaling pathway. *Toxicology Letters*, *290*, 1–9. https://doi.org/10.1016/j.toxlet.2018.03.004

Eriksson, J., Koranyi, L., Bourey, R., Schalin-Jäntti, C., Widén, E., Mueckler, M., Permutt, A. M., & Groop, L. C. (1992). Insulin resistance in type 2 (non-insulin-dependent) diabetic patients and their relatives is not associated with a defect in the expression of the insulin-responsive glucose transporter (GLUT-4) gene in human skeletal muscle. *Diabetologia*, *35*(2), 143–147. https://doi.org/10.1007/BF00402546

Feige, J. N., Gelman, L., Rossi, D., Zoete, V., Métivier, R., Tudor, C., Anghel, S. I., Grosdidier, A., Lathion, C., Engelborghs, Y., Michielin, O., Wahli, W., & Desvergne, B. (2007). The Endocrine Disruptor Monoethyl-hexyl-phthalate Is a Selective Peroxisome Proliferator-activated Receptor γ Modulator That Promotes Adipogenesis. *Journal of Biological Chemistry*, *282*(26), 19152–19166. https://doi.org/10.1074/jbc.M702724200

Feige, J. N., Gerber, A., Casals-Casas, C., Yang, Q., Winkler, C., Bedu, E., Bueno, M., Gelman, L., Auwerx, J., Gonzalez, F. J., & Desvergne, B. (2010). The Pollutant Diethylhexyl Phthalate Regulates Hepatic Energy Metabolism via Species-Specific PPARα-Dependent Mechanisms. *Environmental Health Perspectives*, *118*(2), 234–241. https://doi.org/10.1289/ehp.0901217

Gao, D.-W., & Wen, Z.-D. (2016). Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. *Science of The Total Environment*, *541*, 986–1001. https://doi.org/10.1016/j.scitotenv.2015.09.148

Garvey, W. T., Maianu, L., Hancock, J. A., Golichowski, A. M., & Baron, A. (1992). Gene Expression of GLUT4 in Skeletal Muscle From Insulin-Resistant Patients With Obesity, IGT, GDM, and NIDDM. *Diabetes*, *41*(4), 465–475. https://doi.org/10.2337/diab.41.4.465

Genuis, S. J., Beesoon, S., Lobo, R. A., & Birkholz, D. (2012). Human Elimination of Phthalate Compounds: Blood, Urine, and Sweat (BUS) Study. *The Scientific World Journal*, *2012*, 1–10. https://doi.org/10.1100/2012/615068

Habert, R., Muczynski, V., Lehraiki, A., Lambrot, R., LĂŠcureuil, C., Levacher, C., Coffigny, H., Pairault, C., Moison, D., Frydman, R., & Rouiller-Fabre, V. (2010). Adverse effects of endocrine disruptors on the foetal testis development: Focus on the phthalates. *Folia Histochemica et Cytobiologica*, *47*(5), 67–74. https://doi.org/10.2478/v10042-009-0056-5

Harrer, J. M., Ponniah, S., Ferguson, D. G., & Kranias, E. G. (1995). Expression of phospholamban in C2C12 cells and regulation of endogenous SERCA1 activity. *Molecular and Cellular Biochemistry*, *146*(1), 13–21. https://doi.org/10.1007/BF00926876

He, K., Wu, G., Li, W.-X., Guan, D., Lv, W., Gong, M., Ye, S., & Lu, A. (2017). A transcriptomic study of myogenic differentiation under the overexpression of PPARγ by RNA-Seq. *Scientific Reports*, *7*(1), 15308. https://doi.org/10.1038/s41598-017-14275-2

Heckmann, M., Klanert, G., Sandner, G., Lanzerstorfer, P., Auer, M., & Weghuber, J. (2022). Fluorescence microscopy-based quantitation of GLUT4 translocation. *Methods and Applications in Fluorescence*, *10*(2), 022001. https://doi.org/10.1088/2050-6120/ac4998

Heudorf, U., Mersch-Sundermann, V., & Angerer, J. (2007). Phthalates: Toxicology and exposure. *International Journal of Hygiene and Environmental Health*, *210*(5), 623–634. https://doi.org/10.1016/j.ijheh.2007.07.011

Huang, C., Thirone, A. C. P., Huang, X., & Klip, A. (2005). Differential Contribution of Insulin Receptor Substrates 1 Versus 2 to Insulin Signaling and Glucose Uptake in L6 Myotubes*. *Journal of Biological Chemistry*, *280*(19), 19426–19435. https://doi.org/10.1074/jbc.M412317200

Jaldin-Fincati, J. R., Bilan, P. J., & Klip, A. (2018). GLUT4 Translocation in Single Muscle Cells in Culture: Epitope Detection by Immunofluorescence. In K. Lindkvist-Petersson & J. S. Hansen (Eds.), *Glucose Transport* (Vol. 1713, pp. 175–192). Springer New York. https://doi.org/10.1007/978-1-4939-7507-5_14

Jin, Y., Zhang, Q., Pan, J.-X., Wang, F.-F., & Qu, F. (2019). The effects of di(2-ethylhexyl) phthalate exposure in women with polycystic ovary syndrome undergoing *in vitro* fertilization. *Journal of International Medical Research*, *47*(12), 6278–6293. https://doi.org/10.1177/0300060519876467

Kahn, B. B., Rossetti, L., Lodish, H. F., & Charron, M. J. (1991). Decreased in vivo glucose uptake but normal expression of GLUT1 and GLUT4 in skeletal muscle of diabetic rats. *The Journal of Clinical Investigation*, *87*(6), 2197–2206. https://doi.org/10.1172/JCI115254

Karlsson, H. K. R., & Zierath, J. R. (2007). Insulin signaling and glucose transport in insulin resistant human skeletal muscle. *Cell Biochemistry and Biophysics*, *48*(2–3), 103–113. https://doi.org/10.1007/s12013-007-0030-9

Katsikantami, I., Sifakis, S., Tzatzarakis, M. N., Vakonaki, E., Kalantzi, O.-I., Tsatsakis, A. M., & Rizos, A. K. (2016). A global assessment of phthalates burden and related links to health effects. *Environment International*, *97*, 212–236. https://doi.org/10.1016/j.envint.2016.09.013

Kelley, K. E., Hernández-Díaz, S., Chaplin, E. L., Hauser, R., & Mitchell, A. A. (2012). Identification of Phthalates in Medications and Dietary Supplement Formulations in the United States and Canada. *Environmental Health Perspectives*, *120*(3), 379–384. https://doi.org/10.1289/ehp.1103998

Kim, S. H., & Park, M. J. (2014). Phthalate exposure and childhood obesity. *Annals of Pediatric Endocrinology & Metabolism*, *19*(2), 69. https://doi.org/10.6065/apem.2014.19.2.69

Knudsen, J. R., Henriquez-Olguin, C., Li, Z., & Jensen, T. E. (2019). Electroporated GLUT4-7 *myc* -GFP detects *in vivo* glucose transporter 4 translocation in skeletal muscle without discernible changes in GFP patterns. *Experimental Physiology*, *104*(5), 704–714. https://doi.org/10.1113/EP087545

Lapinskas, P. J., Brown, S., Leesnitzer, L. M., Blanchard, S., Swanson, C., Cattley, R. C., & Corton, J. C. (2005). Role of PPARα in mediating the effects of phthalates and metabolites in the liver. *Toxicology*, *207*(1), 149–163. https://doi.org/10.1016/j.tox.2004.09.008

Lauro, D., Kido, Y., Castle, A. L., Zarnowski, M.-J., Hayashi, H., Ebina, Y., & Accili, D. (1998). Impaired glucose tolerance in mice with a targeted impairment of insulin action in muscle and adipose tissue. *Nature Genetics*, *20*(3), 294–298. https://doi.org/10.1038/3112

Lin, Y., Wei, J., Li, Y., Chen, J., Zhou, Z., Song, L., Wei, Z., Lv, Z., Chen, X., Xia, W., & Xu, S. (2011). Developmental exposure to di(2-ethylhexyl) phthalate impairs endocrine pancreas and leads to long-term adverse effects on glucose homeostasis in the rat. *American Journal of Physiology-Endocrinology and Metabolism*, *301*(3), E527–E538. https://doi.org/10.1152/ajpendo.00233.2011

Lind, P. M., Zethelius, B., & Lind, L. (2012). Circulating Levels of Phthalate Metabolites Are Associated With Prevalent Diabetes in the Elderly. *Diabetes Care*, *35*(7), 1519–1524. https://doi.org/10.2337/dc11-2396

Liu, J., Zhou, B., Yan, M., Huang, R., Wang, Y., He, Z., Yang, Y., Dai, C., Wang, Y., Zhang, F., & Zhai, Q. (2016). CLOCK and BMAL1 Regulate Muscle Insulin Sensitivity via SIRT1 in Male Mice. *Endocrinology*, *157*(6), 2259–2269. https://doi.org/10.1210/en.2015-2027

Lizunov, V. A., Stenkula, K. G., Lisinski, I., Gavrilova, O., Yver, D. R., Chadt, A., Al-Hasani, H., Zimmerberg, J., & Cushman, S. W. (2012). Insulin stimulates fusion, but not tethering, of GLUT4 vesicles in skeletal muscle of HA-GLUT4-GFP transgenic mice. *American Journal of Physiology-Endocrinology and Metabolism*, *302*(8), E950–E960. https://doi.org/10.1152/ajpendo.00466.2011

Lottrup, G., Andersson, A.-M., Leffers, H., Mortensen, G. K., Toppari, J., Skakkebaek, N. E., & Main, K. M. (2006). Possible impact of phthalates on infant reproductive health. *International Journal of Andrology*, *29*(1), 172–180. https://doi.org/10.1111/j.1365-2605.2005.00642.x

Marsee, K., Woodruff, T. J., Axelrad, D. A., Calafat, A. M., & Swan, S. H. (2006). Estimated Daily Phthalate Exposures in a Population of Mothers of Male Infants Exhibiting Reduced Anogenital Distance. *Environmental Health Perspectives*, *114*(6), 805–809. https://doi.org/10.1289/ehp.8663

Melnick, R., Lucier, G., Wolfe, M., Hall, R., Stancel, G., Prins, G., Gallo, M., Reuhl, K., Ho, S.-M., Brown, T., Moore, J., Leakey, J., Haseman, J., & Kohn, M. (2002). Summary of the National Toxicology Program's report of the endocrine disruptors low-dose peer review. *Environmental Health Perspectives*, *110*(4), 427–431. https://doi.org/10.1289/ehp.02110427

Miyawaki, J., Sakayama, K., Kato, H., Yamamoto, H., & Masuno, H. (2007). Perinatal and Postnatal Exposure to Bisphenol A Increases Adipose Tissue Mass and Serum Cholesterol Level in Mice. *Journal of Atherosclerosis and Thrombosis*, *14*(5), 245–252. https://doi.org/10.5551/jat.E486

Net, S., Sempéré, R., Delmont, A., Paluselli, A., & Ouddane, B. (2015). Occurrence, Fate, Behavior and Ecotoxicological State of Phthalates in Different Environmental Matrices. *Environmental Science & Technology*, *49*(7), 4019–4035. https://doi.org/10.1021/es505233b

Peijnenburg, W. J. G. M., & Struijs, J. (2006). Occurrence of phthalate esters in the environment of the Netherlands. *Ecotoxicology and Environmental Safety*, *63*(2), 204–215. https://doi.org/10.1016/j.ecoenv.2005.07.023

Planelló, R., Herrero, O., Martínez-Guitarte, J. L., & Morcillo, G. (2011). Comparative effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) on the aquatic larvae of Chironomus riparius based on gene expression assays related to the endocrine system, the stress response and ribosomes. *Aquatic Toxicology*, *105*(1–2), 62–70. https://doi.org/10.1016/j.aquatox.2011.05.011

Radke, E. G., Galizia, A., Thayer, K. A., & Cooper, G. S. (2019). Phthalate exposure and metabolic effects: A systematic review of the human epidemiological evidence. *Environment International*, *132*, 104768. https://doi.org/10.1016/j.envint.2019.04.040

Rajesh, P., & Balasubramanian, K. (2014). Di(2-ethylhexyl)phthalate exposure impairs insulin receptor and glucose transporter 4 gene expression in L6 myotubes. *Human & Experimental Toxicology*, *33*(7), 685–700. https://doi.org/10.1177/0960327113506238

Schreiber, A., Fu, F., Yang, O., Wan, E., Gu, L., & LeBlanc, Y. (n.d.). *Increasing Selectivity and Confidence in Detection when Analyzing Phthalates by LC-MS/MS*. 6.

Song, K. S., Scherer, P. E., Tang, Z., Okamoto, T., Li, S., Chafel, M., Chu, C., Kohtz, D. S., & Lisanti, M. P. (1996). Expression of Caveolin-3 in Skeletal, Cardiac, and Smooth Muscle Cells. *Journal of Biological Chemistry*, *271*(25), 15160–15165. https://doi.org/10.1074/jbc.271.25.15160

Stahlhut, R. W., van Wijngaarden, E., Dye, T. D., Cook, S., & Swan, S. H. (2007). Concentrations of Urinary Phthalate Metabolites Are Associated with Increased Waist Circumference and Insulin Resistance in Adult U.S. Males. *Environmental Health Perspectives*, *115*(6), 876–882. https://doi.org/10.1289/ehp.9882

Sun, Q., Cornelis, M. C., Townsend, M. K., Tobias, D. K., Eliassen, A. H., Franke, A. A., Hauser, R., & Hu, F. B. (2014). Association of Urinary Concentrations of Bisphenol A and Phthalate Metabolites with Risk of Type 2 Diabetes: A Prospective Investigation in the Nurses' Health Study (NHS) and NHSII Cohorts. *Environmental Health Perspectives*, *122*(6), 616–623. https://doi.org/10.1289/ehp.1307201

Swan, S. H., Main, K. M., Liu, F., Stewart, S. L., Kruse, R. L., Calafat, A. M., Mao, C. S., Redmon, J. B., Ternand, C. L., Sullivan, S., Teague, J. L., & the Study for Future Families Research Team. (2005). Decrease in Anogenital Distance among Male Infants with Prenatal Phthalate Exposure. *Environmental Health Perspectives*, *113*(8), 1056–1061. https://doi.org/10.1289/ehp.8100

Swedenborg, E., Rüegg, J., Mäkelä, S., & Pongratz, I. (2009). Endocrine disruptive chemicals: Mechanisms of action and involvement in metabolic disorders. *Journal of Molecular Endocrinology*, *43*(1), 1–10. https://doi.org/10.1677/JME-08-0132

Sylow, L., Tokarz, V. L., Richter, E. A., & Klip, A. (2021). The many actions of insulin in skeletal muscle, the paramount tissue determining glycemia. *Cell Metabolism*, *33*(4), 758–780. https://doi.org/10.1016/j.cmet.2021.03.020

Tang, J., He, A., Yan, H., Jia, G., Liu, G., Chen, X., Cai, J., Tian, G., Shang, H., & Zhao, H. (2018). Damage to the myogenic differentiation of C2C12 cells by heat stress is associated with up-regulation of several selenoproteins. *Scientific Reports*, *8*(1), 10601. https://doi.org/10.1038/s41598-018-29012-6

Tanida, T., Warita, K., Ishihara, K., Fukui, S., Mitsuhashi, T., Sugawara, T., Tabuchi, Y., Nanmori, T., Qi, W.-M., Inamoto, T., Yokoyama, T., Kitagawa, H., & Hoshi, N. (2009). Fetal and neonatal exposure to three typical environmental chemicals with different mechanisms of action: Mixed exposure to phenol, phthalate, and dioxin cancels the effects of sole exposure on mouse midbrain dopaminergic nuclei. *Toxicology Letters*, *189*(1), 40–47. https://doi.org/10.1016/j.toxlet.2009.04.005

Trasande, L., Spanier, A. J., Sathyanarayana, S., Attina, T. M., & Blustein, J. (2013). Urinary Phthalates and Increased Insulin Resistance in Adolescents. *Pediatrics*, *132*(3), e646–e655. https://doi.org/10.1542/peds.2012-4022

Turcotte, L. P., & Fisher, J. S. (2008). Skeletal Muscle Insulin Resistance: Roles of Fatty Acid Metabolism and Exercise. *Physical Therapy*, *88*(11), 1279–1296. https://doi.org/10.2522/ptj.20080018

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Lee, D.-H., Shioda, T., Soto, A. M., vom Saal, F. S., Welshons, W. V., Zoeller, R. T., & Myers, J. P. (2012). Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. *Endocrine Reviews*, *33*(3), 378–455. https://doi.org/10.1210/er.2011-1050

Viswanathan, M. P., Mullainadhan, V., Chinnaiyan, M., & Karundevi, B. (2017). Effects of DEHP and its metabolite MEHP on insulin signalling and proteins involved in GLUT4 translocation in cultured L6 myotubes. *Toxicology*, *386*, 60–71. https://doi.org/10.1016/j.tox.2017.05.005

Watkins, D. J., Sánchez, B. N., Téllez-Rojo, M. M., Lee, J. M., Mercado-García, A., Blank-Goldenberg, C., Peterson, K. E., & Meeker, J. D. (2017). Phthalate and bisphenol A exposure during in utero windows of susceptibility in relation to reproductive hormones and pubertal development in girls. *Environmental Research*, *159*, 143–151. https://doi.org/10.1016/j.envres.2017.07.051

Wong, C. Y., Al-Salami, H., & Dass, C. R. (2020). C2C12 cell model: Its role in understanding of insulin resistance at the molecular level and pharmaceutical development at the preclinical stage. *Journal of Pharmacy and Pharmacology*, *72*(12), 1667–1693. https://doi.org/10.1111/jphp.13359

Ye, J.-M., Doyle, P. J., Iglesias, M. A., Watson, D. G., Cooney, G. J., & Kraegen, E. W. (2001). *Peroxisome Proliferator–Activated Receptor (PPAR)-␣ Activation Lowers Muscle Lipids and Improves Insulin Sensitivity in High Fat–Fed Rats*. *50*, 7.

Yin, N., Liang, S., Liang, S., Hu, B., Yang, R., Zhou, Q., Jiang, G., & Faiola, F. (2018). DEP and DBP induce cytotoxicity in mouse embryonic stem cells and abnormally enhance neural ectoderm development. *Environmental Pollution*, *236*, 21–32. https://doi.org/10.1016/j.envpol.2018.01.035

Zierath, J. R., Krook, A., & Wallberg-Henriksson, H. (2000). Insulin action and insulin resistance in human skeletal muscle. *Diabetologia*, *43*(7), 821–835. https://doi.org/10.1007/s001250051457