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Diazinon exposure activated transcriptional factors CCAAT-enhancer-binding proteins α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) and induced adipogenesis in 3T3-L1 preadipocytes

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ABSTRACT

Environmental chemical exposure could be a contributor to the increasing obesity epidemic. Diazinon, an organophosphate insecticide, has been widely used in the agriculture, and exposure of the general population to diazinon has been reported. Diazinon has been known to induce neurotoxic effects mainly through the inhibition of acetylcholinesterase (AChE). However, its association with dysregulation of adipogenesis has been poorly investigated. The current study aimed to examine the mechanism of diazinon's effect on adipogenesis using the 3T3-L1 preadipocytes combined with a single-cell-based high-content analysis. The results showed that diazinon induced lipid droplet accumulation in a dose-dependent manner. The dynamic changes of adipogenic regulatory proteins and genes were examined at the three stages of adipogenesis (induction, differentiation, and maturation) in 3T3-L1 cells treated with various doses of diazinon (0, 1, 10, 100 μM) using real-time quantitative RT-PCR and Western Blot respectively. Diazinon significantly induced protein expression of transcriptional factors CCAAT-enhancer-binding proteins α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ), their downstream proteins, fatty acid synthase (FASN), acetyl CoA carboxylase (ACC), fatty acid-binding protein 4 (FABP4), lipoprotein lipase (LPL), adiponectin and perilipin in dose and time-dependent manners. Similarly, the adipogenic genes were significantly induced in a dose and time-dependent manner compared to the relative controls. The current study demonstrates that diazinon promotes lipid accumulation and activates the adipogenic signaling pathway in the in vitro model.

1. Introduction

More than 36.5% of adults and 17% of children in the US have obesity or overweight (C. NCHS Data Brief, 2015), and obesity has become a growing health problem and closely associated with a far-ranging adverse effect on health, contributing to morbidity and mortality (Hossain et al., 2007; Allender and Rayner, 2007; Hursting and Dunlap, 2012; McTigue et al., 2014; Buckley et al., 2016a,b). Obesity is a multifactorial disorder, and a susceptible genetic background indeed predisposes to obesity. However, the current rapid rise in the prevalence of obesity appears to be related to gene-environmental interaction (Stanner and Yudkin, 2001; Swinburn et al., 2011). Accumulating evidence suggests that environmental chemicals, such as endocrine disrupting chemicals (EDCs), could be contributing to the development of obesity and associated metabolic disorders (Janesick and Blumberg, 2011; Newbold et al., 2008, 2009; Baillie-Hamilton, 2002; Newbold, 2010; Hugo et al., 2008; Migliarini et al., 2011; Heindel et al., 2015; Wei et al., 2014). EDCs are exogenous compounds that modulate the endogenous hormonal action through disrupting a variety of metabolic signaling pathways. Prenatal exposure to hexachlorobenzene (DDE, metabolites of pesticide DDT), and polychlorinated biphenyls (PCBs) were found to be associated with increased BMI and weight gain at an early age of children (Verhulst et al., 2009; Codru et al., 2007). Epidemiologic studies have shown that exposures to PCBs, dioxins, and phthalates were closely associated with the prevalence of diabetes (Everett et al., 2007; Deierlein et al., 2016). Occupational exposure to organochlorine and organophosphate (OP) insecticides have been shown to be associated with diabetes (Montgomery et al., 2008).

Diazinon is a widely applied OP insecticide in the US and the worldwide (USEPA, 2006), approximately 13 million pounds of the active ingredient diazinon are used annually on agricultural sites (EPA,

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2016). The wide usage of diazinon in agriculture is likely to pose adverse effect to human from exposure, through multiple pathways in both agricultural and non-agricultural communities. Although diazinon's residential uses were banned in the US in 2004, diazinon residue has still been detected in the agricultural worker, general populations, and ecological risks, due to its wild usage in fruit and vegetable production (EPA, 2016; ATSDR, 2008a). For example, diazinon residue has frequently been found in surface waters or drinking water (Aggarwal et al., 2013), and transported from the site of application by precipitation, fog, and wind to other areas, and has the potential to migrate through the soil and into groundwater (EPA, 2008). In addition, the metabolite of diazinon, 2-isopropyl-4-methyl-6-hydroxypyrimidine, has been detected in urine from 82% of American adults (Hill Jr et al., 1995; MacIntosh et al., 2001). Although the metabolites of diazinon in urine were below the limit of detection in adults, it was detected in 95% of children aged 6–11 years and the non-Hispanic community at 1.45 and 1.49 μg/L, respectively, in the NHANES 2001–2002 (CDC, 2009). Diazinon can be transferred to the developing fetus if the pregnant women used OP containing products (Whyatt et al., 2003). In addition, Occupational workers, farmworkers and their children had up to 10 fold higher of urinary metabolites than that of the NHANES survey during agricultural seasons (Thompson et al., 2014). Importantly, pesticides were found to be tracked into homes of farmworkers where children were highly exposed through take-home pesticide pathway (Coronado et al., 2006). Diazinon can be oxidatively degenerated to diaxozon, much more toxic than diazinon. Diazinon exposure can lead to various adverse health outcomes, not only limit to developmental neurotoxicity through acetylcholinesterase inhibition (Flaskos, 2012; Munoz-Quezada et al., 2013; Yen et al., 2011), but also induction of genotoxicity (Boussabbeh et al., 2016; Jones et al., 2015) and DNA damage (Kashanian et al., 2008). Recently, more studies have raised concerns regarding a multitude of toxic effects of OPs at the sub-toxic dose (Song et al., 1997; Crumpton et al., 2000a; Crumpton et al., 2000b). Interestingly, exposure to diazinon has been shown to cause the rise of incidence of acute pancreatitis (Roeyen et al., 2008; Harputluoglu et al., 2003; Hsiao et al., 1996), and reproductive disorder (Adamkovicova et al., 2014; Chiu et al., 2015), which is associated with endocrine and metabolic-related diseases (Sadr-Azodi et al., 2013; Lowenfels et al., 2009). In addition, Ear-tags impregnated with diazinon have been widely used to effectively control ectoparasites on livestock. The cattle that wearing these ear tags gained significant weight, a net increase of 60% over 5 months as opposed to only a 28% weight gain in cattle without treating ear tags (Spradbery and Tozer, 1996; Maciel et al., 2015). An animal study also suggested that subclinical doses and repeated exposure of diazinon promoted weight gain predominately (Baconi et al., 2013). The further results showed that neonatal rats with low-dose of diazinon not only produced developmental neurotoxicity, but also have lasting effects on metabolism, and these metabolic defects were exacerbated when diazinon exposure occurred at a critical developmental stage (Adigun et al., 2010a,b; Slotkin, 2011). This phenomenon was further reinforced by impaired energy metabolism of the gut microbiome in mice treated with Diazinon (Gao et al., 2017).

To date, a few studies have investigated the mechanisms of insecticides on lipid and glucose metabolism, which ultimately contribute to weight gain, development of obesity, and related chronic metabolic diseases (Kim et al., 2014; Valvi et al., 2012). Given the significance of adverse health effects and widespread use of diazinon, it is of great interest to understand the interplay between adipogenesis and diazinon at a subtoxic dose. In our previous study, we have established the impact of environmental exposure Benzyl butyl phthalate on adipogenesis using an in vitro model combined with high-content analysis. A cell-based high-content analysis emerges as a highly informative approach to analyze the regulation and dynamic changes at a single cell level (Yin et al., 2016). In the current study, we investigated whether diazinon regulates adipogenic signaling pathway and promotes adipogenesis using a multiparametric high-content analysis, suggesting an essential role of diazinon in adipogenesis.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), antibiotics (penicillin and streptomycin), fetal bovine serum (FBS), and 0.25% trypsin were purchased from GE Healthcare Life Sciences (Logan, Utah). Insulin, dexamethasone (DEX),3-isobutyl-1- methylxanthine (IBMX), protease inhibitor cocktail, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Diazinon (Cas: 33-41-5,) was purchased from Chem Service with a purity of 99.2% (West Chester, PA). The name of International Union of Pure and Applied Chemistry (IUPAC) is *O*,*O*-Diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate.

2.2. 3T3-L1 cell culture

3T3-L1 mouse preadipocytes were kindly gifted from Dr. Clifton Bailey's laboratory at the University of Georgia. Cells were maintained in DMEM composed of high glucose, 10% FBS and 100 U/mL penicillin and streptomycin in a 37 °C, 5% CO_2 humidified environment. The cultured cells were maintained in a sub-confluent condition and change of media every 2–3 days.

2.3. 3T3-L1 differentiation and treatments

3T3-L1 cells were cultured to 100% confluence (M1 medium: DMEM containing 10% FBS) in a 12-well plate, 35 mm dish, or 96 well-plate, for RNA, protein, or high-content analysis, respectively. This time point is denoted as day 0. After post-confluence, cells were then incubated in adipogenic induction medium (M2 medium: DMEM containing 1 μM DEX, 0.5 mM IBMX, 167 nM insulin and 10% FBS) for two days, and then cultured in adipogenic differentiation medium (M3 medium: DMEM containing 167 nM insulin and 10% FBS) for another two days, followed by DMEM with 10% FBS (M1) for another four days. The cells cultured with DEX in the M2 medium were used as a positive control, in order to ensure the cell's ability to differentiate into the adipocyte. To examine the effects of diazinon on adipogenesis of 3T3-L1 cells, diazinon was added to M1, M2 without DEX, and M3 media as indicated doses for total 8 days. Cells in the vehicle (DMSO 0.05%) in

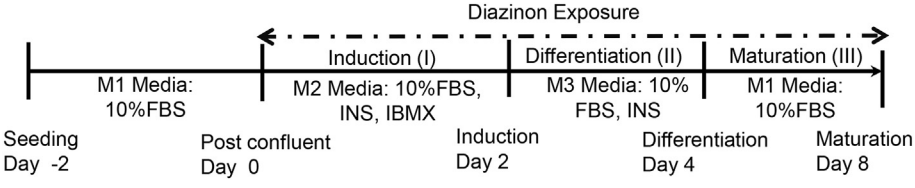

Fig. 1. Illustration of treatment schedule and maturation stages of 3T3-L1 preadipocytes. 3T3-L1 cells were cultured to 100% confluence in 10% FBS DMEM (M1 medium), denoted as day 0. To differentiate the pre-adipocyte 3T3-L1 cells as a positive control (Pos), cells were incubated with 1 μM DEX in adipogenic induction medium (M2 medium: DMEM containing, 0.5 mM IBMX, 167 nM insulin and 10% FBS) for 2 days (day 2). To examine the effect of a compound on the adipogenesis, diazinon was added to the M2 medium for two days, M3 medium for two days, and M1 medium for another four days. Cells cultured in the vehicle (DMSO 0.1%) in all medium was set as the untreated control (CTL). The cellular differentiation was processed into three stages: induction (day 0–2), differentiation (day 2–4) and maturation (day 4–8).