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Rotenone induces reductive stress and triacylglycerol deposition in C2C12 cells

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ABSTRACT

Environmental rotenone is associated with Parkinson's disease due to its inhibitory property to the complex I of mitochondrial respiration chain. Although environmental pollution has been postulated as a causal factor for the increasing prevalence of obesity, the role of rotenone in the pathogenesis of obesity has not been studied. We employed muscle-derived cell C2C12 as a model and shotgun lipidomics as a tool for lipid analysis and found that treatment with rotenone led to the profound deposition of intracellular triacylglycerol (TAG) in a time- and dose-dependent fashion. The TAG deposition resulted from complex I inhibition. Further studies revealed that rotenone induced mitochondrial stress as shown by decreased mitochondrial oxygen consumption rate, increased NADH/NAD+ ratio (*i.e.*, reductive stress) and mitochondrial metabolites. We demonstrated that rotenone activated fatty acid *de novo* synthesis and TAG synthesis and ultimately resulted in intracellular TAG deposition. These studies suggested that increased mitochondrial stresses might be an underlying mechanism responsible for TAG accumulation manifest in obesity.

1. Introduction

Rotenone, a naturally-occurring substance derived from the roots of tropical plants, has been used in fisheries for centuries (Finlayson et al., 2012). It has also been used as a broad spectrum of insecticide, piscicide, and pesticide (Dhaouadi et al., 2010; Patel, 2011). The Farming and Movement Evaluation study (Tanner et al., 2011) has linked rotenone to Parkinson's disease (PD). Animals treated with rotenone have been widely used as models for PD research (Hoglinger et al., 2006). Rotenone inhibits the transfer of electrons from iron-sulfur centers to ubiquinone in the complex I of mitochondrial respiration chain (Gondal and Anderson, 1985). Accordingly, rotenone is recognized as a specific inhibitor of complex I and the majority of actions of rotenone stem from complex I inhibition.

Mitochondria are organelles that produce ATP *via* oxidative phosphorylation. The key component of this process is the electron transport chain (ETC) which consists of four complexes, including NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome bc1 complex (complex III), and cytochrome c oxidase (complex IV). NADH produced by catabolism is oxidized by mitochondrial ETC complex I to yield NAD^+. Two electrons removed from NADH are passed down the ETC and four protons are pumped to the mitochondrial inter-membrane space forming a proton gradient for synthesis of ATP by ATP synthase complex (complex V). Mitochondria also participate in many other intracellular processes, including signaling transduction (Lakshminarasimhan and Steegborn, 2011), biosynthesis (Moraes et al., 2004), cell cycle and death control (Vlachos et al., 2007), and Ca^{++} homeostasis (Wallace, 2007). In addition to PD, mitochondrial dysfunction is also associated with a plethora of chronic conditions, such as heart failure (Rosca and Hoppel, 2010), Alzheimer's disease (Moreira et al., 2010), aging (Guarente, 2008), diabetes (Lowell and Shulman, 2005; Petersen et al., 2003; Ritov et al., 2010; Wang et al., 2010), and obesity (Unger, 2002).

The prevalence of obesity is steadily increasing in the world. The onset of obesity is linked with the increased risk of other life-threatening diseases, such as type II diabetes mellitus (T2DM), cardiovascular disease, and cancer (Bordeaux et al., 2006). It is well known that ectopic triacylglycerol (TAG) accumulation in muscle, liver, and other non-adipose organs, a phenomenon known as lipotoxicity, causes obesity complications such as fatty liver, insulin resistance, and T2DM (Unger, 2002; Unger and Orci, 2000). Both genetic and environmental changes contribute to the etiology of obesity (Silventoinen et al., 2010). Environmental insults

Abbreviations: ACL, ATP citrate lyase; ETC, electron transport chain; FA, fatty acid; GPAT, glycerol-3-phosphate acyltransferase; OCR, oxygen consumption rate; PD, Parkinson's disease; T2DM, type II diabetes mellitus; TAG, triacylglycerol; TCA, tricarboxylic acid.

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that promote obesity include excess caloric intake, reduced physical activity, sleep deprivation, and xenobiotics and environmental toxins (pesticides, herbicides, and industrial chemicals) in our food chains (Trasande et al., 2009).

The impact of mitochondrial complex I malfunction on obesity is not fully understood. A previous study has shown that decreased ETC complex I activity is associated with obesity (Niemann et al., 2011) and lipid accumulation in skeletal muscle has been observed in patients with complex I deficiency (Watmough et al., 1990). ETC complex I activity is significantly reduced in obese or T2DM subjects (Ritov et al., 2010), and in *ob*/*ob* fatty mice (Garcia-Ruiz et al., 2010). However, it is unclear if the environmental rotenone contributes to the increasing prevalence of obesity.

Herein, we showed that rotenone profoundly induced TAG accumulation in muscle-derived cell culture. Mechanistic studies demonstrated that rotenone (1) induced mitochondrial stresses, including decreased mitochondrial oxygen consumption rate (OCR), increased ratio of NADH/NAD^+ (*i.e.*, reductive stress) and mitochondrial metabolites, (2) activated mitochondrial metabolite shuttling into cytoplasm for fatty acid synthesis, and (3) induced TAG synthesis and deposition. Taken together, our results may reveal the relationship between environmental rotenone and obesity.

2. Materials and methods

2.1. Materials

Power SYBR Green PCR master mix, Turbo DNase, and high-capacity cDNA reverse transcription kits were ordered from Applied Biosystems (Frederick, MD); NADH/NAD^+ assay and triglyceride quantification colorimetric/fluorometric kits were purchased from Biovision (Milpitas, CA). Trizol reagent, cell culture medium (*i.e.*, DMEM) and supplements, precast Tris-glycine polyacrylamide gels, and polyvinylidene fluoride membranes were obtained from Invitrogen (Carlsbad, CA). Phosphatase and proteinase inhibitor cocktail tablets (PhosSTOP and Complete Mini) were obtained from Roche Applied Science (Indianapolis, IN). Restore plus Western blot stripping buffer and bicinchoninic acid protein assay kit were from Thermo Fisher Scientific (Rockford, IL). Antibodies against β-actin and phosphorylated ATP citrate lyase (p-ACL) (Ser455) were purchased from Cell Signaling Technology (Boston, MA). JC-10 mitochondrial membrane potential assay kit was purchased from Abcam (Cambridge, MA). The siRNA transfection reagent was obtained from Epoch Life Science (Sugar Land, TX). The antibodies against GPAT (glycerol-3-phosphate acyltransferase) and NDUFV1, and siRNA and control siRNA of NDUFV1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Cell lines (C2C12, H9C2, 3T3-L1, Hepa1-6, HEK293, and BE(2)-C) were obtained from ATCC (Manassas, VA). Piericidin A was obtained from ENZO Life Sciences (Farmingdale, NY). Rotenone and L-carnitine hydrochloride were purchased from Sigma (St. Louis, MO). Oil-Red-O was obtained from Electron Microscopy Sciences (Hatfield, PA). XF cell culture microplates and XF96 extracellular flux assay kits were purchased from Seahorse Bioscience (North Billerica, MA). Other common supplies and chemicals were purchased from Thermo Fisher Scientific or Sigma.

2.2. Cell culture

Cells including C2C12, 3T3-L1, H9C2, Hepa1-6, HEK293, and BE(2)-C were cultured in high glucose DMEM supplement with 10% serum. The medium was changed to serum-free medium when cells reached 80% confluence for 24 h prior to treatment. Cells were treated with or without a complex I inhibitor in low glucose medium for 48 h prior to being harvested for biochemical assays.

2.3. Determination of oxygen consumption rate

C2C12 cells (1×10^4) were plated in the Seahorse 96 well culture plate and treated with different concentrations of rotenone in DMED as indicated for 48 h after serum starved for 24 h. Prior to loading the plate in an XF96 analyzer and measurement of OCR, cells were washed with PBS (warmed at 37 °C) and incubated in Seahorse assay medium at 37 °C for 1 h.

2.4. SiRNA transfection

C2C12 cells were cultured in serum-free medium for 24 h and transfected with siRNA overnight following manufacturer's instruction. The transfection medium was changed to serum-free DMEM. Cells were incubated for 48 h prior to being harvested for biochemical assays. All experimental samples were kept on ice unless specified.

2.5. NADH assay

Cells were lysed in an NADH assay buffer and cell debris was pelleted. Aliquots of cell extracts were used to assay for NAD^+ and NADH with an NADH/NAD^+ quantification kit following manufacturer's instruction.

2.6. Western blot analysis

Western blot analysis was performed as described previously (He, 2010).

2.7. Oil Red-O staining

Cells were plated onto Lab-Tek chamber slides and treated with a complex I inhibitor as described above. Cells were washed with PBS and fixed with 3.7% formaldehyde. The accumulated lipid droplets were stained with Oil-Red-O and the nuclei were counterstained with hematoxylin. Images were acquired with Olympus IX71 inverted microscopy.

2.8. Shotgun lipidomics analysis

Cells were scraped in PBS and cell pellets were kept at −80 °C prior to processing for lipid analysis. Lipid extraction and analysis by multi-dimensional mass spectrometry-based shotgun lipidomics were conducted as previously described (Cheng et al., 2007; Yang et al., 2009). Specifically, identification and quantification of TAG species by using triheptadecenoylglycerol (T17:1 TAG) as an internal standard were performed as previously described (Han and Gross, 2001).

2.9. Real-time RT-PCR

Total RNA isolation from C2C12 cells with Trizol reagent was performed following manufacturer's protocol. RNA (2 μg) was reverse-transcribed into cDNA in 20 μl reaction buffer using a high-capacity cDNA reverse transcription kit following manufacturer's instruction. For real-time PCR, the products of the reverse transcription reaction (2 μl) were amplified using SYBR Green dye (SA Biosciences, CA) along with the primers in an Eppendorf RealPlex2. Target mRNA levels were determined using the ΔΔCt method as described (Winer et al., 1999) using β-actin as a normalizer and expressed as fold changes relative to that of controls. Primers include glycerol-3-phosphate acyltransferase (GPAT) forward 5′-AGCAAGTCCTGCGCTATCAT-3′ and reward 5′-CTCGTGTGGGTGATTGTGAC-3′; β-actin forward 5′-CTGGATGG-CTACGTACATGG-3′ and reward 5′-CTGGATGGCTACGTACATGG-3′.