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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 of rotenone led to the profound deposition of intracellular triacylglycerol (TAG) in a time- and dose-dependent fashion. The TAG deposition was resulted from complex I inhibition. Further studies revealed that rotenone induced mitochondrial stress including 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.

Keywords

ATP citrate lyase; mitochondrial complex I; obesity; shotgun lipidomics; triacylglycerol

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

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Author contribution

QH acquired data, analyzed and interpreted data, and drafted the manuscript. MW and CP acquired data, analyzed and interpreted data. SJG and XH designed the study, analyzed and interpreted data, and critically revised the manuscript. All authors approved the final version of the manuscript.

(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 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 DMEM 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 if not clarified.

2.5. NADH assay

Cells were lysed in an NADH assay buffer and cell debris was pelleted. Aliquots of cell extracts were used for 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 haematoxylin. 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 $\Delta\Delta C_t$ 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'-CTGGATGGCTACGTACATGG-3' and reward 5'-CTGGATGGCTACGTACATGG-3'.

2.10. Analysis of acetyl carnitine and organic acids

Cells were treated with a complex I inhibitor for 48 h and scraped in PBS. A small portion of the cell suspension was saved for protein assay. For analysis of acetyl carnitine, cells were resuspended in 300 µl of ice-cold 50% acetonitrile and 0.3% formic acid while for analysis of organic acids, cells were resuspended in 1 ml of ice-cold 0.1 M HCl and mixed by vortexing until a homogeneous mixture was obtained without remnant of visible cell pellets. The samples were kept on ice at all times. The cell debris was spun down and the cell extract was spiked with premixed isotope-labeled internal standards including sodium L-lactate-3,3,3- d_3 , sodium pyruvate- $^{13}C_3$, sodium D-3-hydroxybutyrate-1,3- $^{13}C_2$, succinic- $^{13}C_4$ acid, fumaric-2,3- d_2 -acid, DL-malic acid-2- ^{13}C , and α -ketoglutaric acid, disodium salt (1,2,3,4- $^{13}C_4$). The extraction was conducted with ethyl acetate and the organic acids were derivatized with BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) as described (Petucci et al., 2012). For measurement of acetyl carnitine mass, cells were incubated in the presence of 1 mM L-carnitine for the last 24 h.

2.11. Measurement of mitochondrial membrane potential

Cells were plated onto 96-well cell culture plate and treated with 5 nM rotenone for 48 h. Cells were washed with PBS and assayed for mitochondrial membrane potential following the manufacturer's instruction. The mitochondrial membrane potential was expressed as the ratio of fluorescence intensity detected at 525 and 590 nm.

2.12. Miscellaneous

Protein concentration was determined utilizing a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) using BSA as a standard. All data are presented as the means $\pm$ SEM of at least three separate experiments as indicated. Statistical significance was determined by a two-tailed student t-test in comparison to control, where $^*p < 0.05$ and $^{#}p < 0.01$.

3. Results

3.1. Rotenone induced TAG deposition in vitro

C2C12 cells (a muscle-derived cell line) treated with 5 nM rotenone for 48 h exhibited profound accumulation of intracellular TAG as determined by shotgun lipidomics (Fig. 1A and B). The TAG deposition was also confirmed by Oil-Red-O staining (Fig. 1C and D). The rotenone-treated cells displayed perinuclear oil droplets compared to the control-treated cells which displayed no oil droplets. Quantified results from shotgun lipidomics showed that rotenone induced intracellular TAG accumulation occurred in a time- and dose-dependent fashion (Fig. 2A). Rotenone induced TAG deposition was also quantitatively confirmed with a triglyceride quantification colorimetric/fluorometric kit from Bivion (data not shown). We further investigated rotenone-induced fat depot in a variety of cell lines including H9C2 (derived from embryonic rat heart), Hepa1-6 (derived from mouse liver), HEK293 (derived from human embryonic kidney), neuroblastoma (BE(2)-C derived from human bone marrow), and preadipocyte (3T3-L1). We observed mild intracellular TAG accumulation in all these examined cell lines after treatment with rotenone for 48 h (Fig. 2B). The profound TAG deposition (5.61% of total lipids after rotenone treatment vs. 0.66% in control) was only found in C2C12. These results revealed that lipotoxicity induced by rotenone was a common feature in a variety of different cell types, but the most profound TAG accumulation was specific to C2C12 cells.

3.2. Rotenone induced TAG deposition via complex I inhibition

It is well established that the toxicity of rotenone is to inhibit mitochondrial ECT complex I (Xiong et al., 2012). To investigate if complex I inhibition is the causal factor for TAG accumulation induced with rotenone, C2C12 cells were treated with piericidin A, another complex I inhibitor (Darrouzet et al., 1998). Similar to the treatment with rotenone, substantial amounts of TAG accumulated in cells after 48 h treatment (Fig. 3A). This observation was further corroborated *via* specific knockdown of a complex I subunit, NDUFV1, with siRNA. We found that NDUFV1 siRNA effectively decreased NDUFV1 protein levels (Fig. 3B) and led to significant accumulation of TAG in the treated cells in comparison to control treated with scramble siRNA (Fig. 3C). The effects of NDUFV1 knockdown on TAG deposition were lesser than complex I inhibitors. This is most likely due to siRNA knocking down mitochondrial protein at lower efficiency. Taken together, these results indicate that rotenone induced TAG deposition in C2C12 *via* complex I inhibition.

3.3. Rotenone induced mitochondrial stress

The inhibition of complex I may cause mitochondrial fuel overload so that increases mitochondrial burden. Consistent with the property of its complex I inhibition, we found that rotenone reduced mitochondrial OCR (Fig. 4A) and mitochondrial membrane potential (Fig. 4B). Since complex I is the NADH feeding point to the ETC, as expected, we found that rotenone significantly increased the NADH/NAD⁺ ratio compared with control (Fig. 4C). The increased NADH/NAD⁺ ratio also affects mitochondrial redox state resulting in mitochondrial reductive stress.

Acetyl-CoA is the major 2-carbon unit metabolite used for ATP production through TCA cycle and ETC in the mitochondria. Our result showed that rotenone significantly led to the elevation of acetyl carnitine contents (Fig. 4D), which mainly represents the mitochondrial acetyl-CoA level (Noland et al., 2009). Other TCA cycle intermediates were also increased after treatment with rotenone, including fumarate, malate, and α -ketoglutarate (Fig. 4E). In summary, our study demonstrated that rotenone induced mitochondrial stress, including the increased NADH/NAD⁺ ratio, acetyl carnitine, and TCA intermediates.

3.4. Rotenone activated ATP citrate lyase

There is no doubt that significant stress onto the mitochondria result from the increased NADH/NAD⁺ ratio, acetyl-CoA mass, and anaplerosis. One of the means to reduce the mitochondrial stress is to shuttle the increased mitochondrial metabolites into cytoplasm *via* ATP citrate lyase (ACL). The ACL product (i.e., acetyl-CoA in cytoplasm) is used for fatty acid synthesis. Activation of ACL is the first step of fatty acid synthesis (Sun et al., 2010). We found that ACL was activated after treatment with rotenone in a dose-dependent fashion as represented by its phosphorylation (Fig. 5A). Lipidomics analysis provided further evidence for the increased fatty acid *de novo* synthesis (Fig. 5B). Specifically, analysis of the fatty acyl chain mass and composition in TAG pools showed that palmitoleic and oleic acids, representing the predominant newly-synthesized FAs, were mostly increased in the increased TAG of the rotenone treated cells. In contrast, the main storage FA in TAG (i.e., linoleic acid) was not changed (Fig. 5B). We did not observe any significant changes on the major cellular membrane components, such as phospholipids and sphingolipids (data not shown) after treatment with rotenone. This indicates that the accumulated TAG was largely from *de novo* synthesis rather than converted from other lipids. It should be pointed out that recent studies showed that cells could scavenge more fatty acids from serum-containing culture media at stress conditions (Kamphorst et al., 2013, Young et al., 2013). This should not be the case in our experimental settings as the cells were serum starved for 24 h before the treatment of rotenone and cultured in serum-free medium during the treatment.

3.5. Rotenone induced TAG synthesis

The increased fatty acid *de novo* synthesis provided the essential building blocks for TAG synthesis. To further support our notion, we determined the expression levels of glycerol phosphate acyltransferase (GPAT), a rate-limiting enzyme for TAG synthesis and found significant elevation of GPAT expression in cells after treatment with rotenone (Fig. 6A). Consistent with the elevated mRNA level, GPAT protein content was increased as well (Fig. 6B). These results further supported the finding that rotenone induces TAG deposition.

4. Discussion

In the current study, we showed that rotenone induced significant intracellular TAG accumulation *via* inhibition of mitochondrial ETC complex I. TAG accumulation followed complex I inhibition with rotenone was further substantiated by other reagents, including pharmacological inhibitor piericidin A for complex I and siRNA knockdown of complex I subunit NDUFV1. The trigger for the increased FA *de novo* synthesis and TAG accumulation was the mitochondrial stress resultant from ETC complex I inhibition. This is highly specific to muscle-derived cells. To the best of our knowledge, it is demonstrated for the first time that rotenone induced TAG deposition in cultured muscle-derived cells.

These novel findings are highly relevant to the current obesity/diabetes epidemic for several reasons. First, mutation of proteins in ETC complex I that compromises activity, even very modestly, could chronically lead to lipotoxicity. The incidents of such kinds of mutations are expected to be high since mitochondrial ETC complex I is very complex, containing 45 protein subunits in mammals. It is known that mutations crucial to the ETC complex I function are lethal, (e.g., NDUF56 to lethal neonate) (Kirby et al., 2004). Second, individuals who are chronically exposed to the toxins inhibiting ETC complex I function have the potential to elicit obesity. The widely used herbicide atrazine (Lim et al., 2009) which also inhibits complex I may contribute to the increasing prevalence of obesity. Third, mitochondrial dysfunction has already been postulated as a major causal factor for fat accumulation (Bournat and Brown, 2010, Petersen et al., 2004, Vankoningsloo et al., 2006).

It appears to be involved, for example, lipotoxicity in diabetic heart and TAG accumulation in the myoclonic epilepsy with ragged red fibers syndrome (Shoffner et al., 1990).

Our results are consistent with the study showing that inhibition of ETC complexes causes fat accumulation in preadipocytes (Vankoningsloo et al., 2005) and complex I deficiency leads to accumulation of lipids in skeletal muscle (Watmough et al., 1990). Our finding of TAG accumulation in preadipocytes differed from another study (McKay et al., 2003) which showed that rotenone treatment reduced fat mass in the differentiated preadipocytes. There are two key differences between these studies. First, inducing medium containing growth factors and other bioactive cofactors in the previous study was used to test fat mass in the differentiated adipocyte (McKay et al., 2003) whereas low glucose, serum-free medium containing no growth factors was used in our study. Second, the rotenone concentrations differed by 1000-fold: 5 nM in the current study vs. 5 μ M in the previous one. We definitely found conspicuous cell death when the cells were treated with higher concentrations of rotenone.

Rotenone rapidly breaks down after exposure to sunlight (Cavoski et al., 2007). The pollution of rotenone in the environment must be minimal. To investigate the effects of rotenone on human obesity, the condition must be very low dose and long term. It is well known that a relatively high dose of rotenone kills cells (Deng et al., 2010). The current dose used to produce PD in animal is very high because such a high dose causes systemic toxicity and mortality (Greenamyre et al., 2010). This may be part of the reason why the rotenone-induced PD model does not show obesity. Moreover, rotenone-induced TAG deposition in muscle needs to be further investigated *in vivo*.

Obesity patients were blamed for being lazy and not exercising regularly. In fact, their moving system, particularly their muscle contractility, was impaired to a certain degree (Shortreed et al., 2009). Deposition of TAG in muscle obviously impairs the ability of muscle contraction. Taken together, our data showing that low-dose rotenone leads to the profound accumulation of TAG in the muscle-derived cells revealed one of the underlying mechanisms responsible for the induction of environmental toxins to the epidemic prevalence of obesity.

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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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Fig. 1.

Rotenone induced triacylglycerol deposition in C2C12. Cells were treated with 5 nM rotenone for 48 h and harvested for TAG analysis. Lipids from the cells were extracted using a modified Bligh and Dyer procedure. Samples were diluted to a final concentration of ~500 fmol/μl by chloroform/methanol/isopropanol (1/2/4, v/v/v) with 4% LiOH to facilitate lithium adduct in neutral loss [268 (internal standard), 228, 252, 254, 256, 278, 280, 282, 284, 304, and 306] scans. The abundant ion peaks including 849.7, 809.7, 811.7, 813.7, 835.7, 837.7, 839.7, 841.7, 861.8, 863.8, 865.8, 867.8, 887.8, 889.8, 891.8, 893.8, 895.8, 913.8, 915.8, 917.8, 919.8, 921.8, 937.8, 939.8, and 941.8 were identified as lithiated TAG species using shotgun lipidomics as previously described (Han and Gross, 2001). The mass spectra (A and B) were displayed after normalization to the internal standard (IS) peak at m/z 849.7. Data represent 4 separate experiments. C2C12 cells were cultured treated with 0 or 5 nM of rotenone for 48 h. To visualize the accumulated intracellular lipid droplets, cells were fixed with 3.7% formaldehyde prior to staining with Oil-Red-O (C and D). The nucleus was stained with haematoxylin. Images represent 3 separate experiments.

Fig. 2.

Rotenone induced triacylglycerol deposition *in vitro*. A. C2C12 cells were treated with different concentrations of rotenone for different intervals as indicated. TAG contents were determined by shotgun lipidomics and expressed as fold increase *vs.* control. Data represent means $\pm$ SEM from 3–5 separate experiments. * $p < 0.05$ and # $p < 0.01$ compared to control. B. Cells including C2C12, H9C2, Hepa1–6, HEK293, BE(2)-C, and 3T3-L1 were treated with rotenone (5, 10, 5, 5, 20, and 5 nM, respectively) for 48 h. The contents of TAG were determined by lipidomics and expressed as fold increases *vs.* controls which were treated with vehicle. The basal TAG levels are (nmol/mg protein): C2C12, 2.45 ± 0.22 ($n = 14$); H9C2, 3.90 ± 0.27 ($n = 3$); Hep1–6, 11.75 ± 0.28 ($n = 3$); HEK293, 24.17 ± 1.80 ($n = 3$); BE(2)-C, 6.60 ± 0.20 ($n = 3$); and 3T3-L1, 17.53 ± 1.23 ($n = 4$). * $p < 0.05$ and # $p < 0.01$ compared to control.

Fig. 3.

Inhibition of complex I increased TAG deposition. Lipids from the following experiments were extracted and analyzed by shotgun lipidomics as described under the section of "Materials and Methods". A. C2C12 cells were treated with 0 (control) or 1 nM (piericidin A) for 48 h. B and C. C2C12 cells were transfected with NDUFV1 siRNA for 48 h prior to being harvested for NDUFV1 protein and TAG analysis. Protein levels of NDUFV1 and TAG mass levels were analyzed by Western blot using antibody against NDUFV1 (B) and determined by shotgun lipidomics (C), respectively.

Fig. 4.

Rotenone induced mitochondrial stresses. A. C2C12 cells ($\sim 10^4$) were plated in a Seahorse 96-well plate and treated with rotenone for 48 h in serum-free medium. Oxygen consumption rate (OCR) was determined with a Seahorse XF96 Analyzer in Seahorse assay medium. Data represent means $\pm$ SEM of three independent determinations and were expressed as percent of control which was the cells treated with vehicle. B. C2C12 cells were plated in 96-well tissue culture plate and treated with rotenone for 48 h. Mitochondrial membrane potential was assayed with a JC-10 kit from Abcam. Data represent means $\pm$ SD of three separate experiments. C. C2C12 cells were treated with rotenone for 6 (open bars) or 12 h (solid bars) at the concentrations as indicated. Cells were harvested in NADH lysis buffer and assayed for NADH and NAD⁺ contents as described under the section of "Materials and Methods". Data represent means $\pm$ SEM from 8 separate experiments. * $p < 0.05$ and # $p < 0.01$ compared to control. C2C12 cells were treated with 0 or 5 nM rotenone for 48 h, and processed for measurement of acetyl carnitine (D) or organic acids (E) as described under the section of "Materials and Methods". Data represent means $\pm$ SEM from 4 separate experiments for both (D) and (E). * $p < 0.05$ and # $p < 0.01$ compared to control. α -KG stands for α -ketoglutarate.

Fig. 5.

Rotenone activated fatty acid synthesis. A. C2C12 cells were treated with different concentrations of rotenone as indicated for 24 h and processed for determination of activated ATP citrate lyase (ACL) with antibody against p-ACL, whereas β-actin was used as loading control. Data represent means ± SEM from 3 separate experiments. [#]*p* < 0.01 compared to control. B. C2C12 cells were treated with 0 (open bars) or 5 (solid bars) nM rotenone for 48 h. Cells were harvested for quantification of triacylglycerol (TAG) mass and fatty acyl composition by shotgun lipidomics as described under the section of “Materials and Methods”. The composition of palmitoleic (16:1), oleic (18:1), and linoleic (18:2) acids in total fatty acyl of TAG were expressed as percent of total fatty acids in TAG. Data represent means ± SEM from 5 separate experiments. [#]*p* < 0.01 compared with control.

Fig. 6.

Rotenone induced GPAT expression. C2C12 cells were treated with 0 (control) or 5 nM rotenone for 24 h and harvested for determination of expression levels of glycerol-3-phosphate acyltransferase (GPAT) by real-time PCR (A) and Western blot (B) analysis, which were expressed as GPAT/β-actin ratios, respectively. Data represent means ± SEM from 4 separate experiments. * $p < 0.05$ compared to control.