Inhibition of Neuronal Cell Mitochondrial Complex I with Rotenone Increases Lipid β-Oxidation, Supporting Acetyl-Coenzyme A Levels*

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Background: Rotenone exposure is associated with Parkinson disease in humans and rodents, although the exact mechanism remains unknown.

Results: Rotenone increased lipid breakdown and glutamine utilization.

Conclusion: Metabolic shifts compensated for impaired energy production in response to rotenone.

Significance: Metabolic abnormalities associated with mitochondrial dysfunction may play an important role in the development of neurodegeneration.

Rotenone is a naturally occurring organic pesticide known to inhibit complex I of the mitochondrial electron transport chain (1). Epidemiological studies have suggested a positive correlation between rotenone exposure and Parkinson disease (PD) in human populations (2). Furthermore, chronic rotenone exposure induces a parkinsonian phenotype in rodents (3, 4).

Despite a known association between rotenone and dopaminergic neuronal damage, the mechanistic cause of neuronal cell death remains unknown.

There is substantial evidence to suggest that mitochondrial dysfunction plays an important role in the development of PD (5–8). Mitochondrial abnormalities have been well documented in PD patients, often coinciding with elevated markers of oxidative stress (9, 10). Rotenone can induce the formation of reactive oxygen species, resulting in oxidative stress, indicating a potential mechanistic link between reactive oxygen species and PD (11, 12). In contrast, it is unlikely that oxidative stress is the sole factor that links mitochondrial dysfunction with PD, as multiple studies have shown that antioxidant supplementation fails to abrogate the progression of PD in humans (13). Therefore, defects in mitochondrial metabolism apart from reactive oxygen species production could be important to the pathogenesis of PD. We initiated this study to further characterize the metabolic perturbations and to expand upon our current understanding of mitochondrial abnormalities that result from complex I inhibition by rotenone using SH-SY5Y cells. Although SH-SY5Y cells are derived from a human neuroblastoma, they are widely used as an in vitro cell model of dopaminergic neurons in PD (14).

Previous findings from our group revealed that biosynthesis of succinyl-CoA is inhibited at low concentrations of rotenone (IC50 = 25 nM), whereas acetyl-CoA is maintained at constant levels in SH-SY5Y cells (15). Additionally, studies conducted with [13C6]glucose revealed that glycolytically derived acetyl-CoA is reduced substantially in response to inhibition of mitochondrial complex I by rotenone (15). These studies have now been expanded to include both medium- and long-chain acyl-CoA species. Furthermore, potential compensatory metabolic alterations for maintenance of acetyl-CoA levels in response to rotenone have been investigated by utilizing stable isotopically labeled fatty acids and stable isotopically labeled glutamine in combination with isotopologue analyses employing MS-based methodology (16–18). Isotopologues are defined as chemical species that differ only in the isotopic composition of their ions.
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Thus, an isotopologue has at least one atom with a different number of neutrons than its major naturally occurring form.

**EXPERIMENTAL PROCEDURES**

Reagents—N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide, 5-sulfosalicylic acid, ammonium formate solution, glacial acetic acid, rotenone, formic acid, [1,2,3,4-

**13**C₄][palmitoylcarnitine, [1,3,4-

**13**C₃][palmitic acid, [1,2,3,4-

**13**C₄][N₂]glutamine, methoxymethylene HCl, pyridine, pentafluorobenzyl bromide, diisopropylethylamine, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. [1,2,3,4-

**13**C₄][N₂]Sodium octanoate was purchased from Cambridge Isotopes (Tewksbury, MA). Optima LC/MS-grade methanol, acetonitrile (ACN), 2-propanol, and water were purchased from Fisher. 2-(2-Pyridyl)ethyl-functionalized silica gel tubes (100 mg/1 ml) were obtained from Supelco Analytical (Bellefonte, PA). DMEM/F-12 medium, FBS, streptomycin, and penicillin were purchased from Invitrogen. Charcoal-stripped FBS was from Gemini Bio Products (West Sacramento, CA). RPMI 1640 medium without pantothenate was purchased from AthenaES (Baltimore, MD). [1,3,4,5-

**13**C₄][N₂]Calcium pantothenate was purchased from Isosciences (King of Prussia, PA). Oasis HLB solid-phase extraction cartridges were from Waters (Milford, MA). SH-SY5Y cells were obtained from the laboratory of Dr. David Lynch (Children’s Hospital of Philadelphia).

Cell Culture and Rotenone Treatments—SH-SY5Y cells were maintained in 1:1 DMEM/F-12 medium supplemented with 10% FBS, 2 mM l-glutamine, penicillin, and streptomycin. Cells were incubated at 37 °C and 95% humidity with 5% CO₂. When the cells had reached ~80% confluence, they were then treated either with freshly prepared rotenone in DMSO (100 nm final concentration) or with a 1% DMSO vehicle control. For isotopic labeling experiments, cells were treated in the presence of media in which the nutrients or precursors of interest were substituted for their isotopic analogs.

LC/Selected Reaction Monitoring (SRM)/MS Analysis of Acyl-CoA Thioesters—All CoAs were separated using a Phenomenex Luna C₃0 reversed-phase HPLC column (2.0 × 150 mm, 5-μm pore size) with 5 mM ammonium acetate in water as solvent A, 5 mM ammonium acetate in ACN/water (95:5, v/v) as solvent B, and ACN/water/formic acid (80:20:0.1, v/v) as solvent C as described previously (19). A linear gradient was run as follows: 2% solvent B for 1.5 min, increased to 25% over 3.5 min, increased to 100% over 0.5 min, held for 8.5 min, and washed with 100% solvent C for 5 min before equilibration for 5 min. The flow rate was 200 μl/min. Samples were analyzed using an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) in the positive electrospray ionization (ESI) mode. Samples (10 μl) were injected using a LEAP autosampler (CTC Analytics AG, Zwingen, Switzerland) and maintained at 4 °C. Data were analyzed using Analyst Version 1.4.1 software (AB SCIEX). The column effluent was diverted to the mass spectrometer from 8 to 23 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage, 5.0 kV; nitrogen as curtain gas, 15 units; ion source gas 1, 8 units; ion source gas 2, 15 units; and collision-induced dissociation gas, 5 units. The ESI probe temperature was 450 °C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. A loss of 507 Da was monitored for each acyl-CoA.

LC/SRM/MS Analysis of Citrate, Isocitrate, and α-Ketoglutarate—The three metabolites were analyzed using an XBridge BEH130 C₁₈ reversed-phase HPLC column (2.0 × 50 mm, 3.5-μm pore size) with 5 mM ammonium acetate in water/ACN/formic acid (40:60:0.1, v/v) as solvent A and with ACN/formic acid (100:01, v/v) as solvent B. A linear gradient was run as follows: 0% solvent B for 1 min, increased to 100% over 5 min, held for 5 min, and decreased to 0% over 1 min before equilibration for 4 min. The flow rate was 500 μl/min. Samples were analyzed using the API 4000 triple-quadrupole mass spectrometer equipped with an autosampler and software as described above. The column effluent was diverted to the mass spectrometer from 2.5 to 13 min. The mass spectrometer operating conditions were as follows: ion spray voltage, 5.0 kV; nitrogen as curtain gas, 15 units; ion source gas 1, 20 units; ion source gas 2, 25 units; and collision-induced dissociation gas, 6 units. The ESI probe temperature was 400 °C, the declustering potential was 80 V, the entrance potential was 10 V, the collision energy was 25 eV, and the collision exit potential was 15 V.

LC/SRM/MS Analysis of Succinate, Fumarate, and Malate—The three diacids converted to pentafluorobenzyl derivatives (see below) were separated using a CHIRALPAK AD-H column (250 × 4.6 mm, 5-μm particle size; Daicel Chemical Industries, Ltd., Tokyo, Japan) at a flow rate of 1 ml/min. Solvent A was hexanes, and solvent B was 2-propanol/methanol (1:1, v/v). The linear gradient was as follows: 1% solvent B for 3 min, increased to 60% over 22 min, held for 4 min, and decreased to 1% over 1 min prior to a 5-min equilibration. The separation was performed at 30 °C, and a post-column addition (0.75 ml/min methanol) was used. MS analysis was conducted on a TSQ Quantum Ultra AM mass spectrometer (Thermo Scientific, San Jose, CA) equipped with an atmospheric pressure chemical ionization source in the electron capture negative ion mode (20). The TSQ Quantum operating conditions were as follows: vaporizer temperature, 350 °C; heated capillary temperature, 300 °C; and corona discharge needle, 30 μA. The sheath gas (nitrogen) and auxiliary gas (nitrogen) pressures were 35 and 10 arbitrary units, respectively. Collision-induced dissociation used argon as the collision gas at 1.5 millitorr.

LC/MS of Palmitoylcarnitine—Acylcarnitine was analyzed by LC/high-resolution MS as described previously (21). LC separations were conducted using a Waters nanoACQUITY UPLC system with 10 mM ammonium formate in water/ACN/formic acid (40:60:0.1, v/v) as solvent A and 10 mM ammonium formate in 2-propanol/ACN/formic acid (90:10:0.1, v/v) as solvent B. A Waters BEH130 C₁₈ column (100 μm × 150 mm, 1.7-μm pore size) was employed for reversed-phase separation. A partial loop injection of 3 μl was made at a flow rate of 1400 nl/min. The linear gradient was run from 30 to 95% solvent B over 30 min. A Thermo Fisher LTQ Orbitrap XL hybrid mass spectrometer was used in positive ion mode at a resolution of 60,000 with a Michrom CaptiveSpray ESI source. The operating conditions were as follows: spray voltage, 4 kV; capillary temperature, 250 °C; and capillary voltage, 35 V.
Short-chain Acyl-CoA Extraction—Extractions were performed as described in detail previously (22). Briefly, cells were lifted manually with a cell scraper, centrifuged at 500 × g for 5 min, and resuspended in 1 ml of ice-cold 10% TCA, and pulse-sonicated for 30 s on ice using a Fisher sonic dismembrator, followed by a 5-min centrifugation at 15,000 × g. The supernatant was transferred to a fresh tube, and the pellet was discarded. The supernatant was purified by solid-phase extraction as follows. Oasis HLB 1-ml (30 mg) solid-phase extraction columns were conditioned with 1 ml of methanol, followed by 1 ml of water. The collected supernatant was applied, washed with 1 ml of water, and finally eluted using three subsequent applications of 0.5 ml of methanol containing 25 mM ammonium acetate. Eluted compounds were dried down under nitrogen and resuspended in 100 μl of 5:5-sulfosalicylic acid. Injections of 10 μl were made for LC/ESI/MS/MS analysis.

Long-chain Acyl-CoA Extraction—Cells were lifted manually with a cell scraper, centrifuged at 500 × g for 5 min, and resuspended in ACN/2-propanol (3:1, v/v) at 550 μl/10-cm² plate. The internal standard prepared (as described below) using stable isotope labeling by essential nutrients in cell culture (SILEC) was added (200 μl), and samples were pulse-sonicated with a probe tip sonicator on ice 30 times for 0.5 s. 250 μl of 100 mM KH₂PO₄ (pH 6.7) was added to the samples, vortex-mixed, and spun down for 10 min at 16,000 × g for 4°C. The supernatant was transferred to a glass tube and acidified with 125 μl of glacial acetic acid. Solid-phase extraction columns were equilibrated with 1 ml of ACN/2-propanol/water/1% acetic acid (9:3:4, v/v) washing solvent. Samples were transferred to the columns, which were washed two times with 1 ml of the washing solvent. The acyl-CoAs were then eluted by washing the columns twice with 500 μl of methanol and 250 mM ammonium formate (4:1, v/v) in glass tubes. After evaporation to dryness under nitrogen gas, the eluates were redissovled in 50 μl of water/ACN (7:3, v/v) containing 5% (w/v) 5-sulfosalicylic acid and transferred to HPLC vials for LC/SRM/MS analysis. For isotopologue analysis, the same extraction was used except that no internal standard was added.

Organic Acid Extraction and Derivatization—Cells were lifted manually with a cell scraper, centrifuged at 500 × g for 5 min, and resuspended in ice-cold methanol/water (4:1, v/v) at 750 μl/10-cm² plate for extraction. Samples were pulse-sonicated on ice for 30 s. Sonicated samples were centrifuged at 16,000 × g for 10 min. The supernatant was then transferred to a clean tube and dried under nitrogen. For citrate, isocitrate, and α-ketoglutarate, dried residues were derivatized with 150 μl of methoxyamine HCl in pyridine (20 mg/ml) at 40°C for 1 h. Samples were then spiked with 50 μl of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide and incubated at 70°C for 1 h. 20 μl of each sample was diluted with 980 μl of ACN and analyzed by LC/ESI/MS. For succinate, fumarate, and malate, dried samples were derivatized with 100 μl of diisopropylethylamine in ACN (2.5:97.5, v/v) and 50 μl of pentfluorobenzyl bromide in ACN (1:4, v/v) at 37°C for 30 min (23). Samples were dried under nitrogen and resuspended in hexane/ethanol (95:5, v/v) prior to LC/SRM/MS analysis.

Palmitoylcarnitine Extraction—Cells were lifted manually with a cell scraper, centrifuged at 500 × g for 5 min, and resuspended in ACN/2-propanol (3:1, v/v) at 550 μl/10-cm² plate, and pulse-sonicated for 30 s on ice using a Fisher sonic dismembrator, followed by a 5-min centrifugation at 15,000 × g. The supernatant was transferred to a fresh tube, and the pellet was discarded. The supernatant was purified by solid-phase extraction as follows. Oasis HLB 1-ml (30 mg) solid-phase extraction columns were conditioned with 1 ml of methanol, followed by 1 ml of water. The collected supernatant was applied, washed with 1 ml of water, and finally eluted using three subsequent applications of 0.5 ml of methanol containing 25 mM ammonium acetate. Eluted compounds were dried down under nitrogen and resuspended in 100 μl of 5% 5-sulfosalicylic acid. Injections of 10 μl were made for LC/ESI/MS/MS analysis.

RESULTS

Rotenone Increases Conversion of Lipids to Acetyl-CoA

Absolute levels of acyl-CoAs (pmol/10⁶ cells ± S.E.) in SH-SY5Y cells after the addition of 100 nM rotenone (n = 4)

| Acyl-CoA | DMSO | Rotenone |
|---------|------|----------|
| C₂₀     | 8.661 ± 0.492 | 8.236 ± 0.967 |
| C₁₈:0   | 0.139 ± 0.012 | 0.074 ± 0.006² |
| C₁₆:0   | 0.022 ± 0.007 | 0.003 ± 0.001¹ |
| C₁₄:0   | 0.055 ± 0.002 | 0.038 ± 0.001² |
| C₁₂:0   | 0.025 ± 0.003 | 0.010 ± 0.001² |
| C₁₀:0   | 0.030 ± 0.001 | 0.023 ± 0.002² |
| C₈:0    | 0.505 ± 0.035 | 0.274 ± 0.045² |
| C₆:0    | 0.889 ± 0.091 | 0.837 ± 0.112 |
| C₄:0    | 0.732 ± 0.049 | 0.635 ± 0.069 |

* p < 0.01 (Student’s two-tailed t-test against the relevant acyl-CoA species from the vehicle control).
* p < 0.05.
* p < 0.001.

Generation of SILEC Internal Standards—Hepa-1c1c7 cells were passaged at least seven times in custom RPMI 1640 medium without calcium pantothenate and containing 10% charcoal-stripped FBS, 100 units/ml penicillin, 100 mg/liter streptomycin, and 2 mg/liter [¹³C₉,¹⁵N]calcium pantothenate as described previously (19, 22). 24 h before extraction, ultralabeling with medium as described above but omitting charcoal-stripped FBS was performed to ensure optimal stable isotope labeling of CoA species. Upon completion of labeling, Hepa-1c1c7 cells were lifted manually with a cell scraper, centrifuged at 500 × g for 5 min, and re-suspended in ACN/2-propanol/water (5:3:2, v/v). Samples were analyzed by stable isotope dilution LC/high-resolution MS.

TABLE 1 Absolute levels of acyl-CoAs (pmol/10⁶ cells ± S.E.) in SH-SY5Y cells after the addition of 100 nM rotenone (n = 4)
medium-chain CoAs were reduced. This suggests that they were utilized to maintain acetyl-CoA levels through β-oxidation. The decreased formation of the borderline medium/long-chain fatty acid C₁₄ CoA was indicative that this also served to maintain acetyl-CoA levels through β-oxidation. The decrease in the levels of the C₄ short-chain CoA was in keeping with our previous observation that there was a significant increase in its downstream metabolite, β-hydroxybutyryl-CoA, after rotenone treatment (see Fig. 3) (15).

Octanoate and Palmitate Oxidation Is Up-regulated in Response to Rotenone—To investigate the metabolic utilization of acyl-CoA species of varying acyl chain lengths, isotopologue analysis was performed in cells treated with either 100 μM [¹³C₄]sodium octanoate (Fig. 2A) or [¹³C₁₆]palmitic acid (Fig. 2B). SH-SY5Y cells were treated with each tracer separately and either DMSO or 100 nM rotenone for 6 h. Short-chain acyl-CoA species were extracted and analyzed for isotopic enrichment by LC/SRM/MS. Both tracers clearly labeled acetyl-CoA in the M⁺2 isotope, indicating incorporation via β-oxidation (Fig. 2). Surprisingly, rotenone treatment approximately doubled the incorporation of both tracers into acetyl-CoA, indicating that fatty acid catabolism played a compensatory role in maintaining acetyl-CoA levels (Fig. 3). This finding indicates that metabolic shifts took place to support acetyl-CoA production to maintain absolute levels despite diminished biosynthesis from glycolysis.

Glutamine Supports Acetyl-CoA Production by Reductive Metabolism—Previous studies have shown that glutamine plays an important role in the maintenance of acetyl-CoA levels in cancer cells (18, 24–26). Additionally, glutamine is the most abundant endogenous free amino acid, with concentrations of 100 μg/ml (683 μM) in serum and plasma (27, 28) and 50 μg/ml (342 μM) in cerebrospinal fluid (27). This makes it a potentially important metabolic precursor for regulating mitochondrial function. To investigate the utilization of glutamine in SH-SY5Y cells, incubations were performed in glutamine-free medium supplemented with 2 mM [¹³C₅,¹⁵N₂]glutamine and either DMSO or 100 nM rotenone for 6 h. Isotopologue analysis of acetyl-CoA showed an ~3-fold increase in the relative incorporation of glutamine into acetyl-CoA in response to rotenone as revealed by increased labeling in the M⁺2 isotope (Fig. 4A). Glutamine metabolism is known to occur by both oxidative and reductive pathways, and cellular stress plays an important role in switching between the two. In particular, mitochondrial impairment and hypoxic conditions lead to a shift to reductive metabolism, whereby isocitrate dehydrogenase reductively carboxylates α-ketoglutarate to form isocitrate, which is then converted to citrate (Fig. 3) (18, 29, 30). The resulting cytosolic citrate is a substrate for ATP-citrate lyase, resulting in the formation of acetyl-CoA and oxaloacetate. The acetyl-CoA is subsequently converted to malonyl-CoA, the precursor to lipid biogenesis (Fig. 3). Although informative, the observed +2 mass shift in acetyl-CoA does not distinguish the metabolic route by which glutamine is incorporated into acetyl-CoA. Utilizing [¹³C₅,¹⁵N₂]glutamine, the same experiment was performed, followed by isotopologue analysis of citrate. As expected from the labeling patterns, oxidative metabolism of glutamine resulted in a +4 mass shift in citrate, whereas reductive metabolism resulted in a +5 mass shift because the glutamine-derived carbon was no longer lost in the formation of succinyl-CoA (Fig. 3). Rotenone treatment resulted in a large increase in the M⁺5 isotopologue of citrate (Fig. 4A), indicating that a significant proportion of glutamine metabolism occurred via reductive carboxylation of α-ketoglutarate (Fig. 3). Furthermore, the observed increase in M⁺3 isotopologues of malate (Fig. 4B) and fumarate (Fig. 4C) was due to the ATP-citrate lyase-mediated cleavage of [¹³C₅]citrate derived from reductive conversion.
carboxylation of $\alpha$-$[^{13}C_5]$ketoglutarate (Fig. 4D) with the intermediate formation of $[^{13}C_4]$isocitrate (Fig. 4E). However, both succinyl-CoA (Fig. 4F) and succinate (Fig. 4G) showed a marked increase in M+4 isotopologues (derived from $[^{13}C_5]$α-ketoglutarate) in response to rotenone (Fig. 3). This showed that although rotenone induced reductive glutamine metabolism, oxidative metabolism also persisted. The increase in M+2 labeling of acetyl-CoA (Fig. 4H) could have arisen from either oxidative or reductive glutamine metabolism (Fig. 3). Taken together, these findings show that both oxidative and reductive glutamine metabolism helped to maintain acetyl-CoA levels in response to mitochondrial complex I inhibition.

Glutamine-derived Acetyl-CoA Supports Lipogenesis—To further understand the metabolic utilization of glutamine-derived acetyl-CoA, long-chain CoAs were extracted from cells treated with either 100 nM rotenone or DMSO and grown in the presence of 2 mM $[^{13}C_5^{,15}N_2]$glutamate. Isotopologue analysis revealed an increase in the incorporation of glutamine into palmitoyl-CoA, represented by an increase in the labeling in M+2, M+4, and M+6 (Fig. 5). This finding indicates that rotenone also increased the relative contribution of glutamine carbons in lipogenesis.

Palmitoylcarnitine Levels Increase in Response to Rotenone—Import of fatty acids for catabolism is important to meet cellular energy demand (18). To examine the effect that rotenone has on fatty acid utilization, absolute quantification of palmitoylcarnitine was performed using stable isotope dilution LC/high-resolution MS. Cells treated with 100 nM rotenone showed a marked 2.5-fold increase in the intracellular levels of palmitoylcarnitine (Fig. 6). This suggests that in addition to lipogenesis of glutamine-derived acetyl-CoA, import of palmitate plays an important role in compensating for complex I inhibition.

DISCUSSION

Mitochondrial defects are thought to play an important role in a wide range of diseases (31). In particular, the complex I inhibitory effects of rotenone have been tied to the onset of a parkinsonian phenotype in both animal models and human studies (2–4). Although much focus has been placed on oxida-
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Previous studies from our group showed that there is a rotenone-dependent decrease in the incorporation of glucose-derived carbon into both acetyl- and succinyl-CoA (15). Furthermore, there is a marked increase in the absolute levels of β-hydroxybutyryl-CoA (EC₅₀ = 75 nM) in contrast to the decrease in succinyl-CoA (IC₅₀ = 25 nM). We had suggested previously that this might result from an increase in β-oxidation of fatty acids as a compensatory metabolic alteration (15). The present study has revealed that rotenone does in fact have profound impacts on lipid metabolism in a neuronal cell culture model. Absolute quantification of acyl-CoA species showed that although acetyl- and palmitoyl-CoA levels were maintained, medium-chain acyl-CoA species decreased substantially in response to rotenone (Fig. 1). In contrast, isotopologue analysis utilizing isotopically labeled octanoate and palmitate revealed that incorporation of both fatty acids into acetyl-CoA was increased significantly in response to rotenone (Fig. 2).

Lipid metabolism is becoming recognized as an important factor in the pathogenesis of neurodegenerative disease. For example, Parkin, an E3 ubiquitin ligase known to be defective in PD patients, has recently been identified as a regulator of lipid uptake and metabolism (37). These findings suggest that alterations in lipid metabolism induced by rotenone could be a mechanistic link to the observed phenotypes of rotenone exposure.

Rotenone-mediated perturbations in lipid metabolism were also accompanied by a shift in glutamine metabolism to a reductive pathway (Fig. 3). This enabled glutamine to help maintain intracellular levels of both acetyl- and palmitoyl-CoA. Furthermore, glutaminolysis occurred through both oxidative and reductive metabolism. Rotenone has previously been shown to induce reductive glutamine metabolism in renal cancer cells (18). Therefore, the present study has provided additional evidence for the reductive carboxylation pathway (17, 18), which has recently become somewhat controversial (38). It was shown recently that isotopic labeling in citrate can occur from glutamine through isotopic exchange without net flux through the reductive pathway (39). This could be due to the reversibility of all the metabolic steps between α-ketoglutarate and citrate (Fig. 3). If the labeled citrate were then further processed by ATP-citrate lyase, it would ultimately lead to the formation of isotopically labeled lipids. Although net reductive flux was not determined in our study, the large increase in M+5
of citrate from \[^{13}C_5\]glutamine indicates an up-regulation of reductive glutamine metabolism in response to rotenone. Despite the possibility of some labeling occurring through isotopic exchange, rotenone clearly increases the contribution of glutamine to acetyl- and palmitoyl-CoA pools, likely through a combination of reductive and oxidative metabolism.

In addition to increased oxidation of lipids and glutaminolysis, complex I inhibition by rotenone also resulted in increased levels of palmitoylcarnitine. This increase suggests that import of fatty acids also plays a compensatory metabolic role, as suggested previously by Fan et al. (38). This would help maintain long-chain acyl-CoA species, which is particularly important for palmitoyl-CoA, a key precursor in sphingolipid biosynthesis (40).

Complex I inhibition by rotenone appears to induce a seemingly futile cycle of lipid breakdown and synthesis from glutamine. Ordinarily, regulation of this process by malonyl-CoA prevents such cycling. Malonyl-CoA generated from the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (Fig. 3) inhibits carnitine palmitoyltransferase I and prevents import of fatty acids into the mitochondria for oxidation. In addition to regulation of carnitine palmitoyltransferase I, malonyl-CoA is a required substrate for the first and rate-limiting step of de novo fatty acid synthesis by fatty acid synthase. High levels of malonyl-CoA therefore result in decreased fatty acid breakdown coupled with increased fatty acid synthesis. Given the increased levels of lipid synthesis from glutamine (Fig. 5), malonyl-CoA would be expected to increase in response to rotenone. Paradoxically, increased levels of palmitoylcarnitine (Fig. 6) suggest that malonyl-CoA levels should decrease upon rotenone treatment. Malonyl-CoA levels in SH-SY5Y cells were below our limit of quantification, so future studies will require the development of more sensitive quantitative methodology to further understand the mechanistic metabolic consequences of rotenone exposure.

Interestingly, many of the medium-chain lipids such as octanoate are able to enter mitochondria independently of the carnitine shuttle system and can therefore bypass the major regulatory actions of malonyl-CoA. For this reason, medium-chain fatty acids can serve as important metabolic precursors for energy production, and indeed, the decrease in medium-chain CoAs observed in response to rotenone indicates that they might be an important nutrient source in response to complex I inhibition.

Previous studies have shown up-regulation of fatty acid import as an important adaptive response to hypoxia to sustain the structural stability of membrane content (41), coinciding with a concomitant switch from glucose- to glutamine-derived acetyl-CoA for de novo lipid synthesis. In agreement with this finding, rotenone mimics many of the effects observed in hypoxia, including decreased glycolytically derived acetyl-CoA (15), increased glutaminolysis for lipogenic purposes (Fig. 5), and increased fatty acid import (Fig. 6). However, we have shown here for the first time that fatty acids are also utilized as a critical compensatory metabolic nutrient for energetic purposes in times of impaired complex I function (Fig. 2).

It is noteworthy that seven molecules of FADH\textsubscript{2} (from the action of acyl-CoA dehydrogenase) arise for each molecule of palmitoyl-CoA, a key precursor in sphingolipid biosynthesis (40).

Acetyl-CoA lies at a critical intersection of glucose and fatty acid metabolism and is known to play an important role throughout a diverse range of cellular processes, including protein acetylation, ketogenesis, mitochondrial energy production, and cholesterol synthesis. Alterations in acetyl-CoA levels would therefore likely impact a wide range of biological functions. Our study has also shown that fatty acids play an important compensatory role in the maintenance of acetyl-CoA levels in response to rotenone. Although acetyl-CoA is maintained in the SH-SY5Y neuronal cell model, the dramatic decrease in succinyl-CoA could have important biochemical implications. Lysine succinylation has been identified as a widely occurring post-translational modification (43). In particular, metabolic enzymes such as isocitrate dehydrogenase are succinylated (43). The functional role of many of these post-translational modifications has not yet been identified, but they are likely to play important regulatory roles in a similar fashion to acetylation of metabolic enzymes by acetyl-CoA. Regulation of metabolic enzymes by metabolic intermediates is rapidly becoming recognized as an important regulatory mechanism and is undoubtedly altered in times of cofactor depletion (22, 44).

It should be noted that our findings do not distinguish between intracellular compartmentalization of metabolite pools such as mitochondrial and cytosolic acetyl-CoA. Characterizing alterations specific to these two cellular compartments would offer an improved understanding of the metabolic alterations resulting from rotenone exposure. For example, identifying differences between IDH1 (cytosolic) and IDH2 (mitochondrial) may shed additional light on mechanistic changes to reductive glutamine metabolism. Various mitochondrial transporters, including the citrate and malate shuttle, likely play an important role in maintaining bioenergetic homeostasis in times of complex I inhibition. Interrogation of these subcellular specific metabolic activities remains to be performed in our model.

Using SH-SY5Y neuroblastoma cells, we have identified previously unknown metabolic abnormalities in response to rotenone. Future efforts should aim to further elucidate a causal mechanistic understanding of how such metabolic adaptations...
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could be protective or pathogenic. Finally, these findings should be verified in a non-transformed cell line, as well as with in vivo models. Utilizing isolated primary dopaminergic neurons will clarify the relevance of our findings in terms of clear adaptations with pathogenic relevance to PD.

In summary, quantitative stable isotope dilution LC/SRM/MS analysis revealed that rotenone decreased the absolute levels of medium-chain acyl-CoAs in neuronal cells. However, acetyl- and palmitoyl-CoA levels were maintained. Using isoforms of medium-chain acyl-CoAs in neuronal cells. However, MS analysis revealed that rotenone decreased the absolute levels of medium-chain acyl-CoAs in neuronal cells. However, MS analysis revealed that rotenone decreased the absolute levels of medium-chain acyl-CoAs in neuronal cells. However, MS analysis revealed that rotenone decreased the absolute levels of medium-chain acyl-CoAs in neuronal cells. However, MS analysis revealed that rotenone decreased the absolute levels of medium-chain acyl-CoAs in neuronal cells. However, MS analysis revealed that rotenone decreased the absolute levels of medium-chain acyl-CoAs in neuronal cells. However, MS analysis revealed that rotenone decreased the absolute levels of medium-chain acyl-CoAs in neuronal cells. 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