Biochemical profiles of rat primary cultured hepatocytes following treatment with rotenone, FCCP, or (+)-usnic acid

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ABSTRACT — The metabolomic profiles of rat primary hepatocytes following treatment with rotenone, FCCP, or (+)-usnic acid were determined using liquid chromatography-mass spectrometry/mass spectrometry and gas chromatography-mass spectrometry. Significant and similar changes in the levels of 283 biochemical metabolites were associated with the three treatments compared with solvent control samples. Overall, the three treatments generated similar global biochemical profiles, with some minor differences associated with rotenone treatment. All three treatments resulted in a shift in energy metabolism as demonstrated by decreased glycogen stores and glycolysis. A reduced antioxidant response was detected in cells following all treatments. In addition, bile acid biosynthesis decreased as a potential consequence of increased oxidative stress by all three treatments. Conversely, rotenone treatment induced a number of changes after 1 hr, which were not detected in FCCP- or (+)-usnic acid-treated samples; these changes were not sustained over time and included increased NAD+ salvage and lysine degradation. In conclusion, these biochemical profiles could provide new insights into the mechanism(s) of mitochondrial toxicity.

Key words: Metabolomics, Rat primary hepatocytes, Mitochondrial toxicity, Energy metabolism

INTRODUCTION

Adenosine triphosphate (ATP) is produced via the respiratory chain through coupled electron transport and oxidative phosphorylation (Nadanaciva et al., 2007). The transport of electrons through complexes I, II, III, and IV of the respiratory chain pumps protons from the mitochondrial matrix into the intermembrane space, causing a shift in the electrochemical potential across the inner membrane (Chaban et al., 2014). This change in electrochemical potential provides energy to drive F1 ATP synthase activity (increased ATP biosynthesis) when protons are pumped back into the matrix through the F0 proton channel (Jonckheere et al., 2012).

The pesticide rotenone and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) inhibit electron transport-mediated ATP synthesis at two distinct steps (Wood et al., 2005; Brennan et al., 2006; Nadanaciva et al., 2007). Electrons shuttle between complexes I and III through the reduction of ubiquinone. Rotenone prevents the transfer of electrons to ubiquinone, and thus blocks electron transport at complex I (Nadanaciva et al., 2007). In contrast, FCCP does not block electron transport, but functions as an ionophore and transports protons across the inner mitochondrial membrane back into the mitochondrial matrix (Brennan et al., 2006). The diffusion of protons back into the mitochondrial matrix by FCCP decreases the electrochemical potential and further diverts protons from ATP synthase, inhibiting the biosynthesis of ATP. (+)-Usnic acid is a common biochemical metabolite produced by lichens (Sahu et al., 2012). It is thought to possess antimicrobial, antiparasitic, and antitumorigen-
ic activities (Campanella et al., 2002; Elo et al., 2007). Furthermore, it was frequently utilized in dietary weight loss supplements, which have been associated with severe clinical hepatotoxicity (Sanchez et al., 2006). A recent study showed that the cytotoxic effects of (+)-usnic acid are due to increased oxidative stress and mitochondrial dysfunction (Han et al., 2004; Sahu et al., 2012). To identify the mechanism(s) leading to mitochondrial toxicity, the global biochemical profiles of rat primary hepatocytes were determined and compared following treatment with increasing doses of rotenone, FCCP, or (+)-usnic acid for 1, 4 and 24 hr.

**MATERIALS AND METHODS**

**Chemicals**

Rotenone, FCCP, and (+)-usnic acid were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). These chemicals were solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich Japan K.K.) and added to medium so that the final concentration of DMSO was 0.5%. All reagents used for cell culture, except ITS+ premix (BD Biosciences, San Jose, CA, USA), were purchased from Invitrogen (Carlsbad, CA, USA).

**Isolation, culture, and treatment of rat hepatocytes**

Hepatocytes were isolated from three F344/DuCrICrj rats (8- to 15-weeks-old), purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). This procedure was approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd., and conducted in compliance with the “Law Concerning the Protection and Control of Animals”, Japanese Law No. 105, October 1, 1973, revised on June 22, 2005. Rat primary cultured hepatocytes were isolated and cultivated, as described previously (Fujimoto et al., 2010). The initial viability of hepatocytes used in this study exceeded 85%. Two-hundred-thousand cells/well and 1 × 10^6 cells/well of viable hepatocytes were plated in a collagen-coated 24-well plate to assess cytotoxicity, and in a collagen-coated six-well plate to assess metabolomics, respectively. After 6 hr of incubation at 37°C in 5% CO2, cells were overlaid with Matrigel (BD Biosciences). After a culture period of 26 hr, sandwich-cultured hepatocytes were treated with the above compounds dissolved with modified William’s E medium whose component was described in Supplement 1, for 1, 4, and 24 hr. At each of these time points, the cells were harvested and stored at -80°C prior to extraction. Cytotoxicity was assessed using the LDH-Cytotoxic Test (Wako Pure Chemical Industries, Osaka, Japan) and CellTiter-Glo Luminescent Cell Viability Assay (Promega, Tokyo, Japan), according to the manufacturer’s instructions (Fujimoto et al., 2010). Based on our historical data, less than 95% of cell viability and less than 80% of ATP content were defined as significant.

**Sample extraction for metabolomic profiling**

The samples were extracted using an automated MicroLab STAR system (Hamilton Company, Salt Lake City, UT, USA) in methanol, containing the recovery standards. The samples were then separated into 3 equal aliquots for analysis in 3 independent platforms as described below.

**Metabolomic profiling platform**

The untargeted metabolic profiling platform employed for this assay was based on a combination of 3 independent platforms: ultrahigh-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). The details of this platform and metabolite identification have been described previously (Evans et al., 2009; Ganti et al., 2012).

**Data imputation and statistical analysis**

After the data were corrected for minor variation resulting from inter-day variation in instrument tuning (Evans et al., 2009), the missing values for a given metabolite were imputed with the observed minimum detection value, assuming they were below the limit of detection. For the convenience of data visualization, the raw area counts for each metabolite were rescaled by dividing each sample value by the median value for the specific metabolite. Furthermore, Spotfire (version 3.3.2, TIBCO Software, Palo Alto, CA, USA) was used to generate heatmaps to visualize the data (fold change and p-value versus the control). Principal component analysis (PCA) was performed for global metabolic profiles using SIMCA-P software version 11.5 (Umetrics, Umea, Sweden). Data were analyzed using JMP (SAS, http://www.jmp.com), a commercial software package, and “R” (http://cran.r-project.org/), a freely available open-source software package. The observed relative concentrations for each metabolite were log-transformed, because, in general, the variance increased as a function of a metabolite’s average response. Welch’s t-tests were performed to compare data between experimental groups. The false discovery rate (FDR) method was used to account for multiple comparisons, and each FDR was estimated using q values.
RESULTS AND DISCUSSION

Cytotoxicity

All three treatments resulted in ATP depletion without a decrease in cell viability (Fig. 1). Rotenone treatment at all doses decreased ATP levels after 1 hr, and at 1 and 3 μM after 4 hr, despite no change in cell viability. However, ATP levels recovered at 24 hr. FCCP treatment at the highest dose decreased ATP levels after 1 hr, and at 0.3 and 1 μM after 4 hr; a decrease in cell viability was also observed at 1 μM after 4 hr. ATP partly recovered at 0.3 and 1 μM after 24 hr. (+)-Usnic acid at 10 and 30 μM decreased ATP levels at all time points, and markedly decreased cell viability at 30 μM after 24 hr. Thus, these compounds have the potential to induce mitochondrial toxicity, especially degradation of ATP production with short-term, but not long-term (24 hr), exposure.

Global biochemical profiles

Significant and similar changes in the levels of 283 biochemical metabolites were associated with the three treatments, particularly at the highest doses, compared with solvent (DMSO)-treated control samples. Furthermore, the three treatments resulted in non-significant changes in many biochemical metabolites, and the changes showed the same trend compared with the control samples. All change data of the identified 283 biochemical metabolites are attached in Supplement 2. Representative biochemical changes are summarized as a heat map in Fig. 2. Conversely, some of the differences associated with rotenone treatment at the 1-hr time point were not observed in FCCP- or (+)-usnic acid-treated samples. In addition, some of the changes observed with FCCP and (+)-usnic acid acid treatment at 24 hr did not occur with rotenone treatment (Fig. 3). Thus, while these three compounds appear to induce similar biochemical profiles, the action mechanism of FCCP and (+)-usnic acid appears to differ from that of rotenone.

Energy metabolism

Mitochondrial toxicant-mediated depletion of glycogen

During the fed state, excess glucose is stored as glycogen in the liver for utilization during periods of fasting or for when a rapid energy source is needed (Magnusson et al., 1994; Roach, 2002). In this study, glycogen breakdown products from maltohexaose to glucose significantly increased in DMSO-treated hepatocytes at 24 hr compared with that at the earlier time points (data not shown). This increase may be due to excess glucose in the medium. A slight increase in these glycogen breakdown products was also detected with the lower doses of FCCP and (+)-usnic acid, and the lowest dose of rotenone at 1 hr, which indicates activation of the glycogen breakdown pathway. On the other hand, the glycogen breakdown products were completely depleted by 24 hr following treatment with the higher doses of all three compounds, which indicates inactivate of the glycogen breakdown pathway. These changes may suggest that glycogen stores were utilized for a rapid energy source at 1 hr and depleted at 24 hr in hepatocytes exposed to

![Fig. 1](image_url)

**Fig. 1.** Cell viability (lines) and ATP levels (bars) at each time point. The data represent the mean ± standard deviation of three independent assays. Cell viability (indicated with lines) was assessed by the leakage of lactate dehydrogenase (LDH) from damaged cells. Cell viability was calculated as 0 and 100% following treatment with 0.25% Triton X-100 and solvent (DMSO) control, respectively. The ATP content (indicated with bars) was calculated as the rate to the DMSO control. Dagger and asterisk represent less than 95% of cell viability and less than 80% of ATP content, respectively.
rotenone, FCCP, and (+)-usnic acid. This is likely due to an increased need for glucose as an energy source resulting from the inhibition of ATP biosynthesis through the mitochondrial electron transport chain. The similar changes in glycogen breakdown products observed with (+)-usnic acid and the other two compounds, may suggest that (+)-usnic acid also inhibits ATP biosynthesis mediated by the mitochondrial electron transport chain as reported (Han et al., 2004).

**Decreased glucolysis coincides with glycogen depletion**

An almost identical pattern as that found for glycogen breakdown products at 24 hr was observed for glycolytic intermediates (glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate, and phosphoenolpyruvate) over time following treatment with increasing doses of the three compounds (Fig. 2). These changes in glycolytic intermediates in the DMSO-treated samples demonstrated the ability of cells to enhance glycolysis at 24 hr, which coincided with increased glycogen breakdown. The inability of cells to enhance glycolysis after 24 hr treatment was likely due to the depletion of glycogen stores. Supporting the dependency of rotenone- and FCCP-treated cells on enhanced glycolysis for ATP production, recent studies demonstrated that 2-deoxy-D-glucose (potent inhibitor of glycolysis) alone has little effect on overall ATP levels, but can potentiate the rotenone- and FCCP-mediated reduction in cellular ATP content in hepatocytes (Cheng et al., 2003, Abe et al., 2010).

**Decreased TCA cycle activity**

Levels of the late TCA cycle intermediates fumarate and malate were also significantly lower following rotenone treatment for 24 hr, and FCCP and (+)-usnic acid throughout the time course, with the greatest effect observed following (+)-usnic acid treatment (Fig. 2). Since treatment with (+)-usnic acid resulted in the greatest reduction in glycogen breakdown products and biochemical metabolites associated with glycolysis, the effects of these three treatments on TCA cycle intermediates may also be related to the early depletion of glucose-associated metabolites.

**Oxidative stress**

**Reduced anti-oxidant response**

All three compounds have been demonstrated to increase oxidative stress (Han et al., 2004; Wood et al., 2005; Brennan et al., 2006; Sahu et al., 2012). Supporting a more oxidative environment following rotenone, FCCP, and (+)-usnic acid treatment, the changes in glutathione metabolism were assessed (Fig. 2). Increasing concentrations of all three compounds resulted in a significant decrease in reduced glutathione (GSH), with the greatest effect occurring at 4 hr with rotenone, at 1 and 24 hr with FCCP, and at 4 and 24 hr with (+)-usnic acid (Fig. 2). The lack of change in GSH levels at the earliest time point in Fig. 2. Representative heat map of biochemical metabolites associated with glycogen breakdown, glycolysis, the TCA cycle, glutathione metabolism, and bile acid biosynthesis. This heat map represents fold-change (left) and p-values (right) versus DMSO control samples. Dark blue and red indicate > 2-fold increase and < 0.5-fold decrease, respectively. For p-values, light, neutral, and dark green indicate < 0.1, 0.05, and 0.01, respectively.
rotenone-treated cells may be due to increased recycling of glutathione, as demonstrated by a significant increase in oxoproline at 1 hr (Fig. 2). This significant increase in oxoproline was not detected in FCCP- nor (+)-usnic acid-treated samples. Although oxidized glutathione (GSSG) was significantly decreased when GSH was also decreased in response to the three drugs, increases in cysteine-glutathione disulfide (formed by oxidative stress of glutathione) were also observed (Fig. 2). Thus, the significant decrease in GSH levels, along with increased oxoproline and cysteine-glutathione disulfide levels described above, suggest that cells treated with these three compounds have a reduced ability to respond to an oxidative environment.

Changes in cholesterol and bile acid metabolism

In this study, cholesterol levels were not affected by any of the drug treatments; however, levels of primary bile acids significantly decreased with all three compounds (Fig. 2). Since primary bile acids are produced by hepatocytes, this decrease could be indicative of drug-induced hepatocyte damage or of decreased NADPH levels. Along with reducing oxidized glutathione, NADPH is necessary for the biosynthesis of bile acids (Einarsson et al., 1986). The decrease in primary bile acids could also be related to the increase in 7-beta-hydroxycholesterol. 7-beta-hydroxycholesterol, which is a product of cholesterol oxidation, has been shown to induce apoptosis, deplete cells of glutathione, and function as a surrogate for oxidative stress (Larsson et al., 2007). Increases in oxidized cholesterol would drive cholesterol away from bile acid biosynthesis. Thus, the significant decrease in bile acids observed in hepatocytes

![Fig. 3. Global biochemical profile represented by principal component analysis (PCA) and loading plot. A: PCA at 1 hr, B: loading plot of PCA at 1 hr, C: PCA at 24 hr, D: loading plot of PCA at 24 hr. Diamond, square, circle, and asterisk in A and C indicate rotenone, FCCP, (+)-usnic acid, and DMSO control treatment respectively. Open, grey, and filled symbols in A and C indicate low, middle, and high dose treatment, respectively. Proportions of first (x-axis) and second (Y-axis) principle components at 1 hr were 0.3442 and 0.2177, respectively. Proportions of first (x-axis) and second (Y-axis) principle components at 24 hr were 0.4633 and 0.1368, respectively. Each annotated plot in B and D represents one of the following biochemical metabolites, 1: 2-aminoadipate, 2: N-acetylglutamate, 3: nicotinamide riboside, 4: glutamate.](image-url)
Mitochondrial toxicant-specific biochemical changes

Mitochondrial toxicant-mediated effects on NAD\(^+\) salvage

Nicotinamide adenine dinucleotide (NAD\(^+\)) can be produced through the salvage pathway (nicotinamide and nicotinate as the starting precursors), the de novo pathway (tryptophan as the starting precursor), or through NADH oxidation. Since the only biochemical metabolite associated with de novo NAD\(^+\) biosynthesis detected in this study was tryptophan, the effects of these treatments on the de novo pathway remain to be elucidated. In contrast, the salvage pathway was perturbed in a treatment-dependent manner (Fig. 4). Increasing rotenone concentrations enhanced, but not significantly, the biochemical metabolites associated with the salvage pathway (nicotinamide, nicotinamide riboside, and nicotinamide mononucleotide) at 1 hr, while at subsequent time points, levels of these biochemical metabolites were decreased. In contrast, FCCP and (+)-usnic acid generally decreased levels of biochemical metabolites associated with the salvage pathway at all time points, with the exception of inconsistent changes in nicotinamide across the three FCCP and (+)-usnic acid doses (Fig. 4). While these changes suggest differential effects of the drugs on NAD\(^+\) salvage associated biochemical metabolites, there were no significant changes in NAD\(^+\) levels at any time points in response to any of the drugs tested. Rotenone treatment increases the NADH:NAD\(^+\) ratio due to its effects on mitochondrial electron transport chain complex I (Hull and Whereat, 1967; Wang et al., 1999). As NAD\(^+\) is required for glycolysis, the changes seen in the salvage pathway at 1 hr with rotenone treatment may reflect increased sal-

![Diagram](image)

**Fig. 4.** Schematic of NAD\(^+\) salvage and box plots of salvage pathway-associated biochemical metabolites detected in this study. Asterisk represents statistical significance (p < 0.05) versus the DMSO control samples.
vage to compensate for complex I inhibition. Inhibition of complex I by rotenone would likely result in any NAD⁺ produced being converted to NADH, and would explain why NAD⁺ levels were unaffected by rotenone at this time point. The significance of changes in salvage-associated biochemical metabolites for FCCP or (+)-usnic acid at all time points, or for rotenone at later time points, is unclear, since no changes were observed in NAD⁺ levels. Nevertheless, these differences in salvage-associated biochemical metabolites following FCCP or (+)-usnic acid treatment compared with rotenone treatment suggest that the mechanism of action for (+)-usnic acid is different from that of rotenone, and is possible more similar to that of FCCP.

**Lysine degradation is enhanced by rotenone at 1 hr**

Rotenone resulted in significant changes in 2-aminoadipate and N-acetylglutamate levels (Figs. 3B and 5). The levels of these two biochemical metabolites were increased significantly in a dose-dependent manner up to 7- and 2.7-fold respectively. Both of these biochemical metabolites, along with glutamate (also significantly increased by rotenone) are associated with increased lysine degradation. Lysine degradation occurs in the mitochondrial matrix, resulting in the production of

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**Fig. 5.** Schematic of lysine degradation and box plots of biochemical metabolites associated with lysine degradation. Asterisk represents statistical significance (p < 0.05) versus the DMSO control samples.
acetyl-CoA, as shown in Fig. 5 (Scislowski et al., 1994). Acetyl-CoA can then feed into the TCA cycle for energy production. As with NAD$^+$ salvage, increased lysine degradation may represent an immediate and acute response to energy depletion following rotenone treatment. The effects of rotenone on lysine degradation demonstrated another difference between rotenone and FCCP or (+)-usnic acid, suggesting that FCCP and (+)-usnic acid may have a similar mechanism of action.

**Conclusion**

Treatment with high-dose rotenone, FCCP, and (+)-usnic acid resulted in the rapid expenditure of glycogen stores in primary hepatocytes. Depleted glycogen stores with the high-dose treatments resulted in an inability to feed glycolysis and the TCA cycle at 24 hr, to the extent detected in DMSO-treated samples or following treatment with lower doses of these compounds. These changes in glucose metabolism with FCCP and rotenone likely reflect the inability to produce ATP through oxidative phosphorylation. Thus, the cells become immediately dependent on increased glycolysis for ATP biosynthesis.

In addition to the affected energy metabolism, these compounds enhance the oxidative environment in the cells. Unlike FCCP and (+)-usnic acid, rotenone was also able to acutely increase NAD$^+$ salvage, possibly to compensate for elevated NADH levels, along with lysine degradation; however, rotenone could not sustain these increases over the duration of the experiment. These differences between rotenone and FCCP suggest that compensatory signals are induced by blocking complex I. The lack of effect of FCCP or (+)-usnic acid on the pathways affected by rotenone, along with increases in three unidentified biochemical metabolites (mentioned above), suggests that (+)-usnic acid acts more like an uncoupling agent rather than blocking complex I of the respiratory chain. The changes detected in this study may provide new insights for the identification of metabolites that may induce hepatotoxicity via inhibition of the respiratory chain or that act as uncoupling agents.

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**Conflict of interest**—The authors declare that there is no conflict of interest.
Metabolomics analysis of hepatocytes exposed to mitochondrial toxicants

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