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Assessment of usnic acid toxicity in rat primary hepatocytes using ^{13}C isotopomer distribution analysis of lactate, glutamate and glucose

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Abstract

The lichen metabolite usnic acid (UA) has been promoted as a dietary supplement for weight loss, although cases of hepatotoxicity have been reported. Here we evaluated UA-associated hepatotoxicity *in vitro* using isolated rat hepatocytes. We measured cell viability and ATP content to evaluate UA induced cytotoxicity and applied ^{13}C isotopomer distribution measuring techniques to gain a better understanding of glucose metabolism during cytotoxicity. The cells were exposed to 0, 1, 5 or 10 μM UA concentrations for 2, 6 or 24 h. Aliquots of media were collected at the end of these time periods and the ^{13}C mass isotopomer distribution determined for CO_2 , lactate, glucose and glutamate.

The 1 μM UA exposure did not appear to cause significant change in cell viability compared to controls. However, the 5 and 10 μM UA concentrations significantly reduced cell viability as exposure time increased. Similar results were obtained for ATP depletion experiments. The 1 and 5 μM UA doses suggest increased oxidative phosphorylation. Conversely, oxidative phosphorylation and gluconeogenesis were dramatically inhibited by 10 μM UA. Augmented oxidative phosphorylation at the lower UA concentrations may be an adaptive response by the cells to compensate for diminished mitochondrial function.

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Conflict of Interest

The authors declare that there are no conflict of interest.

Keywords

Usnic acid; Hepatotoxicity; U-¹³C₆-D-glucose; Mass isotopomer distribution analysis (MIDA)

1. Background

Usnic acid (UA) is a dibenzofuran metabolite produced by many lichens, a symbiosis between a wide variety of fungal species and photosynthetically active algae or cyanobacteria. It has been reported that UA has multiple biological effects, including, but not limited to, anti-bacterial, anti-viral, anti-mycotic, anti-protozoal, anti-proliferative, anti-inflammatory and anti-pyretic (Ingolfssdottir, 2002; Guo et al., 2008). Given these activities, lichens have been in the pharmacopeia of many cultures for a host of ailments (Ingolfssdottir, 2002). In general, the mechanisms of action for the above effects are not fully understood, and most have not been pursued clinically.

The Dietary Supplement Health and Education Act of 1994 (DSHEA), states that the dietary supplement manufacturer is responsible for ensuring that a dietary supplement is safe before it is marketed. FDA is responsible for taking action against any unsafe dietary supplement product after it reaches the market by issuing recalls and warnings to protect the public. UA is a dietary supplement that was on the market before 1994 and therefore it is grandfathered. Despite the lack of pharmacological and safety characterization in the public domain, oral UA has been available in the United States as a dietary supplement to aid in weight loss. Since UA has been on the market, the US Food and Drug Administration (FDA) has received 21 reports of liver toxicity related to the ingestion of dietary supplements that contained UA. This prompted the FDA to issue a warning about one such supplement, *LipoKinetix*, in 2001 (<http://www.cfsan.fda.gov/~dms/ds-lipo.html>). Subsequently, usnic acid and *Usnea barbata* herb were nominated by the National Toxicology Program (NTP) for toxicity evaluations (NTP, 2005). Expediently determining the safety profile of UA is a pressing matter because of its availability and continued use.

Several mechanistic studies of UA-related hepatotoxicity have been performed. In cultured primary mouse hepatocytes, usnic acid caused mainly necrosis with no apparent apoptosis (Han et al., 2004). In animal studies, UA induced extensive liver necrosis in mice (Ribeiro-Costa et al., 2004; da Silva Santos et al., 2006) but appeared less toxic to rats, although mitochondrial swelling and changes in endoplasmic reticulum were observed in rat liver (Pramyothin et al., 2004). The proposed mechanisms for UA-related liver injury include uncoupling of oxidative phosphorylation, inhibition of oxidative phosphorylation, increased oxidative stress, lipid peroxidation and depletion of glutathione (GSH) (Han et al., 2004; Pramyothin et al., 2004). The working model is that disruption of mitochondrial respiratory function and oxidative stress conspire to undermine cell viability. Evidence is rapidly accumulating which suggests that disruption of mitochondrial function is a general mechanism that underlies an increasing variety of organ toxicities (Boelsterli and Lim, 2007; Dykens and Will, 2007; Dykens et al., 2008; Joseph et al., 2009).

Therefore, the aim of this study was to assess the effects of UA on mitochondrial function and cell viability. We employed a technique using stable isotope ¹³C labeled glucose (Lee et

al., 1998; Hellerstein and Murphy, 2004; Turner and Hellerstein, 2005) to determine how glucose metabolism was affected in hepatocytes that are treated with UA. This technique used non-radioactive ^{13}C as a dynamic metabolic tracer in intermediate substrates of various metabolic pathways involved in glucose metabolism. The information obtained through these mass isotopomer distribution analyses enabled us to detect disruptions in glycolysis and TCA cycle activities resulting from UA-induced hepatotoxicity. The use of ^{13}C glucose as a tracer will provide an opportunity to monitor cellular energy usage and storage over time.

2. Materials and methods

2.1. Chemicals and reagents

Usnic acid, dimethylsulfoxide (DMSO), ethyl acetate and all media components used for rat hepatocyte culture were purchased from Sigma–Aldrich (St. Louis, MO). The $^{13}\text{C}_6$ labeled glucose (CLM-1396, U- $^{13}\text{C}_6$, 99%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). The CellTiter-Glo Luminescent Cell Viability Assay and CellTiter 96 Aqueous Cell Proliferation Assay were purchased from Promega (Madison, WI).

2.2. Rat hepatocyte isolation and culture

Sprague–Dawley rats (6–8 week-old) were obtained from the breeding colony of the FDA's National Center for Toxicological Research. Rats were anesthetized with 1.5 ml/kg of Nembutal, which contains 50 mg/ml of pentobarbital sodium, prior to undergoing liver perfusion. All animals used in this study were handled in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Institutes of Health and the experimental procedures were approved by the NCTR Institutional Animal Care and Use Committee (IACUC). Rat primary hepatocytes were isolated by a two-stage collagenase perfusion process (Kreamer et al., 1986; Seglen, 1976). Primary hepatocytes were suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 mg/ml glucose, 10% fetal bovine serum (FCS), 25 mM HEPES, 0.5 U/ml insulin, 0.1 μM dexamethasone, 50 U/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 .

2.3. Cell viability measurement

The cytotoxicity of UA on rat primary hepatocytes was evaluated using tetrazolium reduction cell viability assay (MTS assay, CellTiter 96 Aqueous Cell Proliferation Assay, Promega Corporation). Rat primary hepatocytes were plated in 96-well plates at a cell density of $2 \times 10^4/\text{well}$ in 100 μl media. Cell viability was measured at each time point and dose level three separate times. After being cultured overnight at 37 °C, the DMEM media was changed to serum-free media and cells were treated with either vehicle control (0.5% DMSO) or UA in 0.5% DMSO at the concentrations of 1, 2, 5, 10, 20 and 50 μM . At the indicated time points, culture medium was removed and 20 μl of reconstituted MTS/phenazine methosulfate (PMS) was mixed with 100 μl culture medium (DMEM) and added back into each well, and then incubated for an additional 2 h. The absorbance at 490 nm was measured with a microplate reader (μQuant , Universal Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT, USA). The viability of the cells was expressed as a

percentage by comparing the absorbance of UA treated cells to that of the untreated cells (DMSO controls). Cytotoxicity was evaluated based on the mean from at least three independent 96-well measurements. GraphPad Prism 5 (GraphPad Software) was used to calculate the IC₅₀s. The Sigmodial dose–response equation was used to do all the curve-fitting. The r^2 value, a measurement of goodness of fit, was bigger than 0.8 in each cases.

2.4. Cellular adenosine triphosphate (ATP) level measurement

The cultured rat primary hepatocytes were prepared in a similar manner as described in the cytotoxicity test above. ATP levels were measured three times for each time point and dose. After being cultured overnight, the old medium was replaced with serum-free medium, and cells were treated with UA at the concentrations and time periods specified. The total ATP content in the cells and media was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation) with Multi-mode GloMax (Promega Corporation). The IC₅₀s were calculated by the same method as for MTS assays.

2.5. Study design for stable isotope fluxes of glucose

Freshly isolated primary rat hepatocytes were plated in T-75 Falcon culture flasks at a density of 2×10^5 /ml in 10 ml culture medium. After overnight incubation, the culture medium was replaced with fresh DMEM medium that contained 1.0 mg/ml glucose (unlabeled), where 1.8 mg/ml U-¹³C-glucose tracer and UA at concentrations of 0, 1, 5 or 10 μ M were added to the media. In our experiment the starting glucose concentration was 2.8 mg/ml glucose, of which 1.8 mg/ml (64%) was provided as the [U-¹³C₆]-glucose-tracer. The cells were then incubated at 37 °C for an additional 2, 6 or 24 h. Six replicates from three individual experiments were prepared for each treatment group and exposure time producing a total of 72 samples. The cells were washed twice with 5 ml of pre-warmed 37 °C phosphate-buffered saline (PBS) and then removed from the flasks by trypsinization. Cell pellets were obtained following centrifugation at 14,000g for 5 min. Media and pellet samples were frozen at –80 °C until analysis.

2.6. ¹³CO₂ analysis

One hundred microliters of sample media was transferred to gas chromatography (GC) vials, followed by 50 μ l of 0.1 M NaHCO₃ (Mallinckrodt, 7412) and 50 μ l of 1 N HCl (Fisher, 7647-01-0). The vials were immediately capped and the headspace analyzed by GC/MS for CO₂ by monitoring ions m/z 44 and 45 using electron impact selective ion monitoring (SIM) mode.

2.7. Lactate analysis

Sample media (100 μ l) was transferred to microcentrifuge tubes to which two drops of 1.2 N HCl were added to bring the pH to less than 2. One ml of ethyl acetate (Sigma HPLC grade) was added and the mixture vortexed for 15 s. The contents were allowed to settle and the upper ethyl acetate layer was transferred to a glass screw top tube. The extract was carefully dried under nitrogen at a pressure of 5 psi. Residual lactate was then derivatized to lactate *n*-propylamide *n*-heptafluorobutyrate using conditions similar to those described by Tserng et. al. (1984).

The lactate derivative was dissolved in 150 μl of dichloromethane and transferred to GC vials with 250 μl inserts. The samples were analyzed by GC/MS using methane Chemical Ionization (CI) and monitoring ion signals m/z 328, 329, 330 and 331 in SIM mode. These ions are representative of the M0, M1, M2 and M3 ^{13}C isotopomers of lactate, respectively (Marin et al., 2004).

2.8. Glucose analysis

Sample media (100 μl) was transferred to a microcentrifuge tube and deproteinized by the addition of 500 μl of both 0.3 N barium hydroxide (Sigma, B4059-500ML) and 0.3 N zinc sulfate (Sigma, Z2876-500ML). The mixture was vortexed and then centrifuged at 9000g for 15 min. The supernatant was transferred to a glass screw capped culture tube and dried under a gentle stream of nitrogen at or slightly above ambient room temperature using an Organomation Associates (OA-SYS) Heating System (11880-MULTIVAP).

The extracted glucose was derivatized to glucose aldonitrile pentaacetate using methods much like that described by Magni et al. (1992). In brief, hydroxylamine hydrochloride (3 mg) in 150 μl of pyridine was added to the dried extract samples and heated at 100 $^{\circ}\text{C}$ for 2 h. After this, 100 μl of acetic anhydride 99% was added and the contents heated for an additional 1 h at 100 $^{\circ}\text{C}$. Samples were then dried under a gentle stream of nitrogen. The residue was dissolved in ethyl acetate (200 μl) and transferred to vials with 250 μl inserts for GC/MS analysis. Glucose ^{13}C isotopomer distribution was determined by monitoring the ion clusters at m/z 242 (carbon 1–4, top half of glucose) and at m/z 187 (carbons 3–6, bottom half of glucose) using electron impact (EI) selective ion monitoring (SIM).

2.9. Glutamate analysis

Cell culture media (100 μl) was centrifuged and extracted. An equal volume of deionized water was added and the mixture was frozen at -80°C for 1 h. The samples were then centrifuged at 300g for 30 min at room temperature to thaw just before extraction.

The supernatant was transferred to glass screw capped culture tubes and dried under nitrogen. Glutamate was derivatized to its *N*-trifluoroacetyl di-*n*-butyl ester (TAB-glutamate) under conditions like those previously described (Roach and Gehrke, 1969). The derivative was dissolved in 200 μl of methylene chloride and transferred to vials with 250 μl inserts for GC/MS analysis.

Under EI conditions, ionization of unlabeled TAB-glutamate produces two fragment ions, m/z 198 and m/z 152, which correspond to the C2–C5 and C2–C4 fragments, respectively (Bartnik et al., 2007). The ^{13}C isotopomer distribution of glutamate was determined by monitoring the ion clusters m/z 198, 199, 200, 201, and 202 (carbon 2–5 of glutamate) and m/z 152, 153, 154, and 155 (carbons 2–4 of glutamate).

2.10. Gas chromatography/mass spectrometry

Mass spectral data were acquired using an Agilent Technologies' 7890A Gas Chromatograph with a 5975C inert XL MSD Triple-Axis Detector. An Agilent J&W Scientific HP-5MS (30 m $\times$ 0.25 mm $\times$ 0.25 μm , 19091S-433) analytical column was used

for glucose, glutamate, CO₂ and lactate analyses. Helium (>99.9999% purity) was used as the carrier gas.

For glutamate analyses, the GC injection port temperature was set at 250 °C. The initial oven temperature was 150 °C. After sample injection, the oven was held at 150 °C for one minute and then increased 3 °C/min to 221 °C, held for 1 min then increased 20 °C/min to 235 °C and held for one min. The transfer line temperature was 280 °C. The MS source and quadrupole temperatures were 230 and 150 °C, respectively.

For CO₂ analysis, the inlet temperature was 120 °C. The oven was held at 80 °C for the first minute and then increased 10 °C/min to 150 °C. The transfer line, source, and quadrupole temperatures were 150, 230 and 150 °C, respectively.

For lactate analysis, the inlet temperature was 200 °C. The oven was initially 50 °C for the first 0.5 min, then increased 10 °C/min to 150 °C, then 40 °C/min to 250 °C and held at that temperature for 5 min. The transfer line, MS source, and MS quadrupole temperatures were: 280, 300 and 150 °C, respectively.

Injection volumes of 1 µL were used, except for the CO₂ analysis, in which case 10 µL of headspace was injected. All injections were made in split mode; typically using a 10:1 split ratio. All analyses were conducted using SIM mode.

Metabolite ¹³C mass isotopomer distributions were measured using methodology developed by Bartnick (Bartnick et al., 2007). For the analysis of CO₂, only ions m/z 44 and 45 were monitored. For glucose, twelve ions were monitored, m/z 186–191 that represent the C3–C6 atoms of glucose and m/z 241–246 ions represent the C1–C4 atoms of glucose (Guo et al., 1992). For glutamate, 11 ions were measured, m/z 151–155 and m/z 197–202; and for lactate, 5 ions were measured, m/z 327–331. The methods correct for natural background ¹³C isotopic abundance present in the analytes being investigated.

2.11. Data extraction and processing

Following sample analysis by GC/MS, the spectral data were extracted using the Agilent GC/MSD ChemStation software. This process involves identifying the appropriate chromatographic peak and then overlaying the individual ion chromatograms collected during the SIM analysis. Drawing a line across the peaks and then subtracting background noise generated by each ion. This process was repeated three times and an averaged for each sample metabolite was generated and stored in “Comma Separated Values” (CSV) format. These CSV files were subsequently converted into Excel format using “ABC Amber Excel Converter” software. The Excel files were then sent to SIDMAP, LLC for final data processing.

The mean total number of ¹³C carbons per mole of substrate is given by $\sum n_i m_i$, where m_i is the fractional molar enrichment of the mass isotopomer containing n_i number of ¹³C carbons (Marin et al., 2004; Bartnik et al., 2007). The enrichment in the individual isotopomers refers to the concentration of the isotopomer species expressed as a molar fraction (e.g., M0, M1, M2, M3 ...), which represents the fraction of molecules that contain 0, 1, 2, 3, ... ¹³C

substitutions, respectively. Further examples of similar calculations can be found in Bak et al. (Bak et al., 2007).

2.12. Pentose phosphate pathway analysis

Calculations of isotopomer distributions through the pentose phosphate pathway were performed in the manner reported by Lee et al. (Lee et al. 1998). In this case, we used U-¹³C-glucose instead of 1,2-¹³C glucose. The M2 lactate isotopomer from U-¹³C-glucose forms in much the same way that M1 lactate isotopomer is formed from 1,2-¹³C-glucose via the pentose phosphate pathway. Therefore, the pentose phosphate pathway isotopomer distribution calculation (PPP) was approximated using the equation in Lee et al. (1998) by substituting M2/M3 for M1/M2, such that the percent flux through the pentose phosphate pathway during glycolysis was calculated by

$$\text{PPP}(\%) = [(M2/M3)/(3+M2/M3)] \times 100 \quad (1)$$

The M2 and M3 lactate content levels are provided in Table 1.

2.13. Statistical analysis

Statistical analyses were performed using the parametric unpaired, two-tailed independent sample *t*-test with 99% confident intervals with respect to control. *P* < 0.05 was considered to indicate significant difference. Data are expressed as mean ± standard deviation.

3. Results

3.1. Cytotoxicity of UA to Rat primary hepatocytes

The viability of rat primary hepatocytes did not appear to be altered significantly by treatment with 1 μM UA at 2, 4, 6 or 24 h (Fig. 1A) although the cell viability was decreasing with longer exposure times. At 2 μM UA the cell viability decreased from 97 ± 11.5% at 2 h to 90.0 ± 4.9% at 24 h. At the 5 μM UA concentration level, cell viability was not appreciably less than control cultures at the 2, 4 and 6 h incubation periods (92.2 ± 10.8%, 90.7 ± 12.1%, and 88.8 ± 7.7%, respectively, but it was decreased at the 24 h incubation periods (45.9 ± 7.3%). At the 10 μM UA concentration level, cell viability vs. control was decreased for all incubation periods (91.7 ± 10.0% at 2 h; 73.3 ± 2.7% at 6 h, and 29.4 ± 12.2% at 24 h). The calculated IC₅₀ was 29, 22, 14, and 2 μM for 2, 4, 6 and 24 h treatments, respectively. These results indicate that UA caused cytotoxicity to rat primary hepatocytes and the cytotoxicity induced by UA is time- and concentration-dependent.

3.2. ATP levels

Cellular ATP levels of rat primary hepatocytes following UA exposure for 2, 4 and 24 h are presented Fig. 1B. The cellular ATP levels did not appear to be significantly reduced when cells were incubated with 1 μM UA for 2, 4 or 24 h compared to controls (98.9 ± 8.1%, 116.6 ± 5.8, and 98.8 ± 3.9%, respectively). However, all UA treatments beyond 1 μM resulted in large decreases in ATP content. At 2 μM UA concentration, ATP levels were 95.7 ± 4.7%, 107.0 ± 9.9%, and 69.1 ± 12.4% at 2, 4 and 24 h, respectively. For the 5 μM UA

concentration, ATP levels were $88.1 \pm 4.4\%$, $104.0 \pm 12.2\%$, and $31.4 \pm 14.5\%$ at 2, 4 and 24 h, respectively. After treatment with 10 μM UA the ATP levels were almost depleted at 2 h ($17 \pm 5.8\%$), 4 h ($6.1 \pm 2.8\%$) and at 24 h ($1.9 \pm 1.2\%$). The calculated IC_{50} was 7, 6 and 1 μM for 2, 4, and 24 h treatment, respectively. These results indicate that UA caused ATP to deplete in a time- and concentration-dependent manner.

3.3. Media $^{13}\text{CO}_2$ enrichment

Fig. 2 shows the metabolism isotopomer pathways of ^{13}C -U-glucose in hepatocytes and the isotopomers including $^{13}\text{CO}_2$ detected in the media. In general, there was a time-dependent increase in $^{13}\text{CO}_2$ enrichment during the study (Fig. 3). At all time points, $^{13}\text{CO}_2$ enrichments were lower for the 10 μM UA concentration compared to all other treatments but the reduction was not significant at any time point. At 6 and 24 h, both the 1 and 5 μM UA resulted in significantly higher $^{13}\text{CO}_2$ enrichments compared to their respective controls.

3.4. Media lactate ^{13}C content

Similar to $^{13}\text{CO}_2$ enrichment results, there was a time-dependent increase in media lactate ^{13}C content. The total (M1 + M2 + M3) isotopomer lactate content in the media is shown in Fig. 4A. The total lactate ^{13}C content for each treatment increased from 2 to 24 h. At 2 h, the 10 μM UA treatment resulted in a significant decrease in ^{13}C lactate content compared to the respective control. However, at 6 h there was no significant change at any treatment level compared to the respective control. At 24 h, ^{13}C lactate content was significantly higher only at 5 μM UA compared to the control.

3.5. Direct glucose oxidation in the pentose phosphate pathway

The pentose phosphate pathway was calculated using Eq. (1) and the M2 and M3 values shown in Table 1. Fig. 4B shows a concentration-dependent decrease in the percentage of glucose that directly oxidized through the pentose phosphate pathway. At 2 and 6 h, the 5 and 10 μM UA treatments resulted in significantly decreased direct glucose oxidation via G6PDH in the pentose phosphate pathway compared to the respective controls. However, at 24 h, there was no significant change at any UA concentration to alter the rate of direct glucose oxidation in comparison with that of control cells.

3.6. Media glucose isotopomer M1, M2, and M3 fractions

The C1–C4 glucose isotopomer M1, M2, and M3 molar fractions are reported in Fig. 5A–C while the C3–C6 glucose M1 and M3 isotope molar fractions are provided in the supplementary material Fig. 1A–B. Fig. 5A shows a time-dependent increase in the ^{13}C glucose M1 isotopomer fraction. At 2 h, there were no significant differences in ^{13}C glucose M1 isotopomer molar fractions between treatments. At 6 and 24 h, the 10 μM UA resulted in significantly lowered ^{13}C glucose M1 isotopomer fraction compared to their respective controls.

The C1–C4 glucose M2 isotopomer molar fractions are presented in Fig. 5B. The M2 data illustrate a time and dose related decrease as was seen in the M1. However, there are some differences in that at 2 h, both the 5 and 10 μM UA treatments resulted in significantly reduced glucose M2 isotopomer molar fractions compared to the control. The M2 glucose

isotopomer molar fraction at 24 h after treatment with 10 μ M UA was reduced by 85.9% compared to control.

The C1–C4 glucose M3 isotopomer molar fractions are presented in Fig. 5C. At 2 h the 10 μ M UA treatment resulted in a significant decrease in M3 fraction compared to the control. At 6 h the 5 μ M UA and 10 μ M UA treatments resulted in significant decreases in glucose M3 isotopomer molar fractions compared to the respective controls. However, at 24 h, there was no significant change at any treatment level compared to the control.

3.7. Media glutamate M1, M2, and M3 isotopomer fractions

The glutamate M1 isotopomer molar fraction percentage is presented in Fig. 6A. At 2 h, glutamate M1 isotopomer fraction was increased in the 10 μ M UA treatment group, although this did not reach statistical significance compared to the controls. At 6 h, all UA treatments resulted in significantly higher glutamate M1 isotopomer molar fraction compared to the control. At 24 h, only the 10 μ M treatment showed significantly higher glutamate M1 molar fraction relative to control. However, when all three time points are compared, the overall glutamate M1 isotopomer molar fraction pattern exhibited a time-dependant decrease from 2 to 24 h.

Glutamate M2 isotopomer molar fraction percentage is presented in Fig. 6B. At 2 h, the treated groups were not significantly different from controls. At 6 h all the treatments were significantly decreased compared to the control. Both the 5 and 10 μ M UA treatments caused a significant reduction in the glutamate M2 isotopomer molar fraction compared to the control at 24 h. The data in Fig. 6B suggest a time-dependent increase in glutamate M2 isotopomer fraction ^{13}C labeling.

Glutamate M3 isotopomer molar fraction percentage is presented in Fig. 6C. At 2 and 6 h, glutamate M3 isotopomer fraction was decreased in the 10 μ M UA treatment group. At 24 h, glutamate M1 isotopomer fraction was increased in the 1 μ M and 5 μ M UA treatment groups. The data in Fig. 6C suggest a time-dependent increase in glutamate M3 isotopomer fraction percentage labeling.

4. Discussion

The data show that UA is cytotoxic to rat primary hepatocytes in a time- and concentration-dependant manner (Fig. 1A), which is in accord with published data (Han et al., 2004). The depletion of ATP levels in the hepatocytes is hypothesized to occur through uncoupling of mitochondria. ATP was depleted very rapidly and before cell viability was reduced. At the 10 μ M concentration level, the effect of UA on ATP production was swift and profound, resulting in a decrease of ATP levels to 17.0% after 2 h and to <2% of control values after 24 h (Fig 1B). The increased $^{13}\text{CO}_2$ production with the 1 and 5 μ M UA treatments compared to the 10 μ M treatment (Fig 3), suggest that the lower UA concentration caused a shift in metabolism towards oxidative phosphorylation. This is most likely an attempt at the cellular level to ameliorate the uncoupling effects of UA on ATP synthesis. The data, therefore, appear to suggest that the 1 and 5 μ M UA treatments were below the concentration threshold where the cells were still able to adapt by increasing oxidative phosphorylation to partially

overcome the toxic effects of UA, while the 10 μM UA treatment was higher than the threshold capacity to overcome the toxic effects of UA. However, this statement should be interpreted with caution since our longest cell incubation period lasted only 24 h. Longer incubation periods may manifest different results.

The observed total lactate ^{13}C content increase in the study is related to the amount of labeled glucose that was used during glycolysis. This result is consistent with an increased glycolysis that is needed to make up for decreased ATP synthesis at 5 and 10 μM UA concentrations. The percentage of labeled glucose that went through the pentose phosphate pathway was approximated using Eq. (1) and the (M2/M3) ratio shown in Table 1. When the U- ^{13}C -glucose tracer goes directly through glycolysis (Fig. 2) without entering the pentose phosphate cycle, the resulting lactate will have all three carbons labeled (M3). If the U- ^{13}C -glucose tracer enters the pentose phosphate pathway, at least one labeled carbon will be removed upon reentry to glycolysis. Eq. (1) is an approximation to the amount of labeled glucose entering the pentose oxidative and non-oxidative branches versus the amount that directly goes to glycolysis. At 2 and 6 h, the 5 and 10 μM UA concentrations exhibited less labeled glucose entering the pentose phosphate pathway and more labeled glucose going through glycolysis directly.

Decreased direct glucose oxidation in the pentose cycle relative to glycolysis is an important finding for the interpretation of $^{13}\text{CO}_2$ release data in order to determine TCA cycle function in UA exposed primary liver cells. It seems that UA is efficient in increasing TCA cycle turnovers, yet, with decreased ATP yield. While this finding explains UA weight managing effect by increased net carbon loss from carbohydrates via complete oxidation, the decrease in coupled ATP synthesis poses a serious risk to liver cells. Alternate methods yet to be performed that measure oxygen consumption directly in liver cells such as the Seahorse Bioscience methods, may confirm decreased oxygen consumption and thus further clarify a complex-V inhibiting/uncoupling effect of ATP production from complete substrate oxidation in mitochondria in the presence of UA.

The results for the C3–C6 glucose isotopomer fractions are provided in the Supplementary material. The M1 and M3 isotopomer distributions for the C3–C6 glucose are similar to the M1 and M3 isotopomer distributions for the C1–C4 glucose isotopomers but the changes, with respect to controls at each time point, in the C3–C6 glucose are not as pronounced as was observed for the isotopomer distributions for the C1–C4 glucose fragment. A possible reason for this may be due to the fact the C3–C6 portion of glucose is in rapid exchange with glycerol from fats (Chen et al., 2005). The C1–C4 glucose M1 isotopomer is newly formed glucose produced from other substrates via carboxylase in a process known as carbon recycling (Xu et al., 2003). The 10 μM UA treatment severely impaired net glucose M1 production at 24 h in primary hepatocytes. Similarly, the C1–C4 glucose M2 isotopomer is indicative of glucose derived acetate's futile cycling through the TCA cycle, followed by carbon exchanges via the gluconeogenic pathways (Beger et al., 2009). Appearance of glucose M2 isotopomer after 5 and 10 μM UA was reduced at 2 h while the 10 μM UA treatment was severely reduced at 24 h, which points to complete substrate oxidation in the TCA cycle, instead of malate shuttling and M2 glucose production by UA treated cells. The

C1–C4 glucose M3 isotopomer molar fraction pattern, on the other hand, was similar to the pattern of the ^{13}C lactate content and pentose phosphate pathway depicted in Fig. 4A and B.

The differential effects of UA on the cells discussed above are supported by the glutamate data. The ^{13}C labeling pattern of glutamate M1 isotopomer fraction suggests a dose-dependent decrease in the activity of the TCA cycle at 6 and 24 h (Fig 6A). It has been hypothesized that UA induces its effect by uncoupling mitochondrial oxidative phosphorylation (Abo-Khatwa et al., 1996; Pramyothin et al., 2004). This hypothesis is consistent with the observed concentration-dependent trend that shows an increase in glutamate M1 isotopomer fraction at 6 and 24 h, a decrease in glutamate M2 isotopomer fraction from 6 to 24 h time in this study, and a decrease in M3 isotopomer fraction at 2 and 6 h (Figs 6A, 6B and 6C). The increase of M1 glutamate in the media is a sign that acetate is not being completely turned over in the TCA cycle and this data point to a blocked reaction downstream from 2-oxo-glutarate such as the succinate to fumarate reaction. The succinate to fumarate reaction ($\text{succinate} + \text{FAD} \rightarrow \text{fumarate} + \text{FADH}_2$) is a component of the TCA cycle that is directly connected to ATP synthesis. The M3 glutamate isotopomer results from more than one complete TCA cycle. The fact that the M3 glutamate isotopomer is decreased in the 10 μM UA treatment is consistent with acetate not being completely turned over in the TCA cycle at the highest UA treatment.

5. Conclusions

Stable isotope labeling in combination with cell viability and ATP depletion experiments confirm UA-induced toxicity in hepatocytes observed by others (Han et al., 2004; Ribeiro-Costa et al., 2004; da Silva Santos et al., 2006). The observed increase in oxidative phosphorylation at 1 and 5 μM UA may be an attempt by the cells to compensate for diminished mitochondrial function as evidenced by altered carbon dioxide, lactate, glucose and glutamate isotopomer labeling patterns. Although this short term study did not find compromised viability with low concentrations of UA in hepatocyte cultures, its long term use as a nutritional supplement may well pose a risk for hepatocyte longevity. The isotopomer distribution results show oxidative phosphorylation and gluconeogenesis were significantly reduced at the 10 μM UA concentration. These results are consistent with the cytotoxicity and ATP depletion observed in the cells at this concentration. The apparent decrease in complete acetate turnaround in the TCA cycle with a concomitant increase in completely cycled glutamate indicates a block where fumarate is formed from succinate. Whether the blockage in fumarate formation is related to UA-induced proton decoupling or to some other unknown mechanism cannot be determined from this study. The consistency of the isotopomer distribution results demonstrate that the ^{13}C -stable isotope labeling technique is a helpful tool that can be used to better understand oxidative phosphorylation and other energy-related changes that occur during xenobiotic-induced toxicity that are mediated through the mitochondria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.fct.2011.07.047.

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Fig. 1. Hepatocyte cell viability (Fig. 1A) and adenosine triphosphate (ATP) level (Fig. 1B) assessments.

Fig. 2.

Glucose metabolic isotopomer pathways and metabolites detected in the media.

Abbreviations – G6P: glucose –6-phosphate; G1P: glucose-1-Phosphate; F6P: Fructose-6-Phosphate; GAP: glyceraldehyde-3-Phosphate; OAA: oxaloacetate.

Fig. 3. Media $^{13}\text{CO}_2$ enrichment at 2, 6, and 24 h following exposure of rat primary hepatocytes to 0, 1, 5 and 10 μM UA concentrations. * P -value (<0.05) versus 0 μM UA at that time point.

Fig. 4.

The total (M1 + M2 + M3) isotopomer media ^{13}C lactate content at 2, 6, and 24 h following exposure of rat primary hepatocytes to 0, 1, 5 and 10 μ M UA treatments (Fig. 4A). Pentose phosphate pathway flux percentage at 2, 6, and 24 h after exposing rat primary hepatocytes to 0, 1, 5 and 10 μ M UA treatments (Fig. 4B). * P -value (<0.05) versus 0 μ M UA at that time point.

Fig. 5. Media M1 glucose isotopomer fraction percentage (Fig. 5A), media M2 glucose isotopomer fraction percentage (Fig. 5B) and media M3 glucose isotopomer fraction percentage (Fig. 5C) at 2, 6, and 24 h, respectively following exposure of rat hepatocytes to 0, 1, 5 and 10 μM UA concentrations. * P -value (<0.05) versus 0 μM UA at that time point.

Fig. 6. Media M1 glutamate isotopomer fraction percentage (Fig. 6A), media M2 glutamate isotopomer fraction percentage (Fig. 6B), and media M3 glutamate isotopomer fraction percentage (Fig. 6C) at 2, 6, and 24 h following exposure of rat hepatocytes to 0, 1, 5 and 10 μM UA concentrations. **P*-value (<0.05) versus 0 μM UA at that time point.

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Table 1

Media M2 and M3 lactate isotopomer contribution to lactate's ^{13}C labeled fraction at 2, 6, and 24 h for cells treated with 0, 1, 5, and 10 μM UA.

UA Dose (μM)	2 h			6 h			24 h		
	M2	M3	M2/M3	M2	M3	M2/M3	M2	M3	M2/M3
0	12.32	80.37	0.1532	11.89	79.98	0.1487	12.52	78.09	0.1603
1	12.21	80.13	0.1523	12.17	79.32	0.1534	13.04	77.13	0.1691
5	10.08	83.68	0.1204	10.10	82.82	0.1220	13.09	76.67	0.1707
10	11.44	81.12	0.1410	10.81	81.02	0.1334	12.44	77.95	0.1596