J Appl Physiol 122: 1003–1010, 2017.
First published December 22, 2016; doi:10.1152/japplphysiol.00873.2016.

RESEARCH ARTICLE

Melatonin protects against uric acid-induced mitochondrial dysfunction, oxidative stress, and triglyceride accumulation in C_2C_{12} myotubes

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Submitted 29 September 2016; accepted in final form 15 December 2016

Maarman GJ, Andrew BM, Blackhurst DM, Ojuka EO. Melatonin protects against uric acid-induced mitochondrial dysfunction, oxidative stress, and triglyceride accumulation in C_2C_{12} myotubes. *J Appl Physiol* 122: 1003–1010, 2017. First published December 22, 2016; doi:10.1152/japplphysiol.00873.2016.—Excess uric acid has been shown to induce oxidative stress, triglyceride accumulation, and mitochondrial dysfunction in the liver and is an independent predictor of type-2 diabetes. Skeletal muscle plays a dominant role in type 2 diabetes and presents a large surface area to plasma uric acid. However, the effects of uric acid on skeletal muscle are underinvestigated. Our aim was therefore to characterize the effects of excessive uric acid on oxidative stress, triglyceride content, and mitochondrial function in skeletal muscle C_2C_{12} myotubes and assess how these are modulated by the antioxidant molecule melatonin. Differentiated C_2C_{12} myotubes were exposed to 750 μM uric acid or uric acid + 10 nM melatonin for 72 h. Compared with control, uric acid increased triglyceride content by ~237%, oxidative stress by 32%, and antioxidant capacity by 135%. Uric acid also reduced endogenous ROUTINE respiration, complex II-linked oxidative phosphorylation, and electron transfer system capacities. Melatonin counteracted the effects of uric acid without further altering antioxidant capacity. Our data demonstrate that excess uric acid has adverse effects on skeletal muscle similar to those previously reported in hepatocytes and suggest that melatonin at a low physiological concentration of 10 nM may be a possible therapy against some adverse effects of excess uric acid.

NEW & NOTEWORTHY Few studies have investigated the effects of uric acid on skeletal muscle. This study shows that hyperuricemia induces mitochondrial dysfunction and triglyceride accumulation in skeletal muscle. The findings may explain why hyperuricemia is an independent predictor of diabetes.

uric acid; melatonin; oxidative stress; mitochondrial dysfunction

HYPERURICEMIA (elevated serum uric acid) is considered to be an independent risk factor for type 2 diabetes (17). Obese subjects with high levels of uric acid have lower insulin sensitivity and insulin-stimulated glucose disposal and higher levels of oxidative stress compared with obese subjects with normal uric acid concentration (5). Because skeletal muscle plays a dominant role in glucose homeostasis, it is highly likely that it contributes significantly to the observed effects of hyperuricemia. However, studies that have described the effects of uric acid on lipid and glucose metabolism in skeletal muscle cells are limited (16). Zhu et al. (51) showed that hyperuricemia decreases phospho-Akt content and inhibits insulin signaling in mouse skeletal muscle.

Lanaspa et al. (13) recently showed that human HepG2 cells exposed to uric acid exhibited elevated levels of oxidative stress that were associated with lowered mitochondrial membrane potential and decreased mitochondrial aconitase activity. Aconitase (or aconitate hydratase) is an enzyme of the tricarboxylic (TCA) cycle that is sensitive to oxidation by the superoxide anion radical, a reactive oxygen species (ROS). These results support the theory that inhibition of mitochondrial aconitase by ROS promotes de novo lipogenesis by increasing the supply of citrate for making free fatty acids. Consistent with this theory, others have shown that lowering uric acid level with allopurinol (30 mg/kg in drinking water) reduced hepatic triglyceride (TG) content in rats (13) and prevented hepatic steatosis in the Mongolian gerbil (51). In light of these observations in hepatocytes and the lack of corresponding information in skeletal muscle, we investigated whether uric acid exposure to a skeletal muscle cell line (C_2C_{12} myotubes) would increase oxidative stress, induce lipogenesis, and alter mitochondrial function as it does in hepatocytes (13, 14). As a second objective, we investigated whether melatonin, a well-known antioxidant, modulates the effects of uric acid in this cell line.

We report that excess uric acid significantly increased thiobarbituric acid-reactive substances (TBARS, a marker of lipid peroxidation) as well as antioxidant capacity and TG content. It reduced ROUTINE respiration, complex II-linked respiration, and maximal oxygen consumption. Addition of a physiological concentration of melatonin counteracted those effects of uric acid, without a further alteration of antioxidant capacity.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM, D6429), phosphate-buffered saline (PBS, P4417), sterile trypsin-EDTA solution (5 mg/l trypsin and 2 mg/l EDTA in 0.9% NaCl, T4174), uric acid (U2625), melatonin (M5250), antibiotics (10,000 U/ml penicillin and 10 mg/ml streptomycin, P4333), cell culture T25 flasks (25 cm^2, CLS430639), and all substrates and chemicals for high-resolution respirometry, including digitonin, pyruvate, succinate, glutamate, oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), rotenone, antimycin A, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), and ascorbate were purchased from Sigma-Aldrich (Cape Town, South Africa). Horse serum (Hyclone, irradiated donor equine

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serum, US origin) was obtained from Separations (Cape Town, South Africa). The Wako laboratory TG assay kit was obtained from Wako Chemicals (290-63701) and 96-well Greiner microtiter plates from Lasec (Cape Town, South Africa). The white polysorp Nunc microtiter plates for antioxidant capacity were obtained from ANC Amersham.

Cell Culture

Mouse myogenic C_2C_{12} cells (passage 5) were plated at a density of 10^4 cells/cm^2 and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Pen-Strep) in an incubator at 37°C containing 5% CO_2. Cells were grown in this medium to ~50–60% confluence (for 2–3 days) and then switched to differentiation medium consisting of DMEM supplemented with 2% heat-inactivated horse serum and 1% Pen-Strep and cultured for 2 wk until fully differentiated.

Uric Acid and Melatonin Treatment

Differentiated C_2C_{12} myotubes were separated into four treatment groups: control (CON), uric acid (UA; received 750 μM uric acid), uric acid plus melatonin (UA+MEL; 750 μM uric acid + 10 nM melatonin), and melatonin (MEL, 10 nM melatonin). The length of the treatments was 72 h. Treatment length and drug concentrations were based on previous studies (13, 14, 43). Uric acid was dissolved in DMEM, while melatonin was dissolved in absolute ethanol and reconstituted in DMEM. Bottles containing melatonin were covered in foil, as melatonin is light sensitive (18, 24).

Mitochondrial Respiration with High-Resolution Respirometry

Measurement of mitochondrial respiration was performed with a polarographic oxygen sensor in 2-ml glass chambers of an Oxygraph 2K (Oroboros Instruments, Innsbruck, Austria). The amplified signal from the oxygen sensor was recorded on a computer at sampling intervals of 2 s with DatLab acquisition software (Oroboros Instruments). Before all experiments were commenced, calibration of the respirometer was performed at air saturation, 37°C. After treatments, differentiated C_2C_{12} myotubes were washed with PBS and harvested by adding trypsin-EDTA (0.025%) and centrifuging for 5 min at 500 *g* (at room temperature) in medium. One T25 flask yielded an ~4.5-mg pellet that was suspended in 2 ml of MiR05 respiration medium at 37°C according to the recommended protocol (4). After myotubes were pipetted into the two oxygraph chambers, endogenous ROUTINE respiration was read when oxygen flux stabilized (~15 min). Digitonin was added to the chambers (to a final concentration of 10 μg/ml) and allowed to permeabilize the cell membrane for ~15 min. Permeabilization was reflected by a gradual decrease of oxygen flux until a stable level was observed. Pyruvate (to a final concentration of 5 mM), glutamate (10 mM), and malate (0.5 mM) were added to induce LEAK respiration, followed by ADP (2.5 mM) to stimulate complex I-mediated oxidative phosphorylation (OXPHOS). After addition of succinate (10 mM) complex I+II-linked OXPHOS was registered. Addition of oligomycin (2.5 μM) induced another LEAK respiration. Titration with the uncoupling agent CCCP in steps of 0.5 μM increased respiration up to the maximal level, called electron transfer system (ETS) capacity. Addition of the complex I inhibitor rotenone to a final concentration of 0.5 μM indicated maximal flux via complex II electron flow. Next, addition of antimycin A (2.5 μM) induced residual oxygen consumption (4). Finally, titration of TMPD (0.5 mM) and ascorbate (2 mM) to assess complex IV-linked respiration was performed as a proxy for mitochondrial content. Oxygen flux at all respiratory states was normalized to the complex IV flux to correct for variations in cell content in the oxygraph chambers.

Homogenization of Myotubes

In a separate set of experiments, treated and untreated C_2C_{12} myotubes were washed with PBS and trypsinized. Myotubes were suspended in differentiation medium and centrifuged at 1,500 rpm for 3 min at 25°C. The supernatants were removed, and the pellets were suspended in homogenization medium (0.075 M sucrose, 0.225 M mannitol, 0.0001 M EGTA, and 0.03 M Tris·HCl) and homogenized with a Potter-Elvehjem glass-Teflon homogenizer. Homogenized samples were stored at −80°C and later (within 1 mo) analyzed for TBARS, antioxidant capacity, TG content, and protein concentration.

Protein Determination Assay

Protein concentrations of all samples were determined with the Markwell protein determination assay (21). Briefly, 10 μl of each standard (1–100 μg bovine serum albumin) and sample (made up to 100 μl with distilled water) was added to 300 μl of the Markwell reagent, vortexed, and left at room temperature for 15 min. Next, 30 μl of Folin-Ciocalteu phenol reagent (in a 1-to-1 ratio with distilled water) was added to each tube, vortexed, and left at room temperature for 45 min. Absorbance was determined on a spectrophotometer at 660 nm. A standard curve was generated and used to determine the protein concentration of each sample.

Thiobarbituric Acid-Reactive Substances Assay

The TBARS assay was used to measure malondialdehyde (MDA), a marker of lipid peroxidation, based on the Jentzsch method as previously described (11). Briefly, myotube samples were added in the presence of BHT to orthophosphoric acid and heated with thiobarbituric acid. After phase separation, TBARS were measured spectrophotometrically (SPECTRAmaxPLUS-384; Molecular Devices, Labotec Industrial Technologies, Cape Town, South Africa) with a molar extinction coefficient. Absorbance was read at 532 nm, with a microplate data acquisition program (SoftMax Pro 4.8; Molecular Devices, Labotec Industrial Technologies).

Oxygen Radical Absorbance Capacity Assay

The oxygen radical absorbance capacity (ORAC) assay was used to measure the antioxidant capacity of samples as previously described (18). Briefly, homogenate samples were centrifuged, and the supernatant was diluted with phosphate buffer and pipetted into a microtiter plate with phosphate buffer, fluorescein, and 2,2′-azobis-(2-amidinopropane)-dihydrochloride. Readings were recorded spectrofluorometrically at an excitation wavelength of 485 nm and an emission wavelength of 520 nm with a data acquisition program (Advanced Reads, Cary Eclipse WinFLR; SSM Instruments, Set Point Technology, Cape Town, South Africa). Values were expressed as nanomoles per liter of Trolox equivalents.

Triglyceride Content Assay

The Wako Laboratory Assay TG kit was used for the quantification of TG in myotube samples. The kit provided a glycerol standard that was used to prepare the standards (48). Samples or standards were pipetted into the wells of a 96-well Greiner plate in duplicate, and the TG reagent was added and left to incubate for 30 min at room temperature. A microplate data acquisition program (SoftMax Pro 4.8) was used to read the absorbance at 600 nm. A standard curve was generated and used to determine the TG concentration in each sample. The final TG content per sample was expressed as micrograms per microgram of protein.

Statistical Analyses

All statistical analyses were performed with Graph Pad Prism version 7.00 (for Windows; Graph Pad Software, La Jolla, CA). All treatment groups had sample sizes of five or more, and a two-way ANOVA was performed, followed by a Bonferroni post hoc analysis. Significance was defined as $P < 0.05$. Values are expressed as means ± SE.

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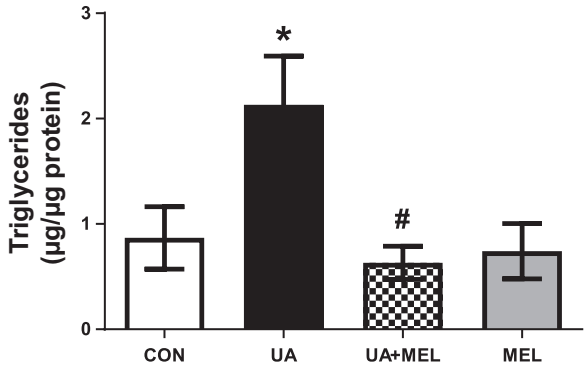

Fig. 1. TG results of C_2C_{12} myotubes exposed to uric acid (750 μM, 72 h) with and without melatonin (10 nM, 72 h) compared with control (CON). Melatonin treatment blocked the increased TG synthesis in the UA group (UA+MEL vs. UA). Data are represented as means ± SE; $n = 6$ per group: $^{*}P < 0.05$ (UA vs. CON); $^{\#}P < 0.05$ (UA+MEL vs. UA).

RESULTS

Triglyceride Content

The effects on TG content of exposing differentiated C_2C_{12} myotubes to 750 μM uric acid and/or 10 nM melatonin for 72 h are shown in Fig. 1. TG content was ~237% higher in uric acid-treated (UA) myotubes compared with controls ($P < 0.05$; UA vs. CON), but when melatonin was administered together with uric acid (UA+MEL) it blocked the increase caused by uric acid alone ($P > 0.05$; UA+MEL vs. CON). Incubation with melatonin alone (MEL) for 72 h did not significantly change TG content (MEL vs. CON).

Lipid Peroxidation and Antioxidant Capacity

A marker of lipid peroxidation, TBARS, was measured as an indication of oxidative stress, and the ORAC was used to estimate antioxidant capacity in C_2C_{12} myotubes after exposure to uric acid and/or melatonin. As shown in Fig. 2*A*, uric acid increased TBARS concentration significantly ($P < 0.005$ vs. CON) whereas MEL alone had no effect ($P > 0.05$; MEL vs. CON). TBARS was not statistically different from CON in UA+MEL myotubes. Relative to control, antioxidant capacity was significantly increased in UA, UA+MEL, and MEL groups ($P < 0.05$ vs. CON; Fig. 2*B*).

Respirometry Measurements

High-resolution respirometry using an Oroboros 2K oxygraph was used to measure ROUTINE respiration in intact C_2C_{12} myotubes and capacities of ETS, OXPHOS, and LEAK in digitonin-permeabilized myotubes supplied with substrates for complex I and complex I+II. Representative oxygraph traces are shown in Fig. 3.

ROUTINE respiration. As shown in Fig. 4, uric acid decreased ROUTINE respiration significantly whereas MEL alone and together with uric acid (UA+MEL) both increased ROUTINE respiration compared with control ($P < 0.05$ vs. CON).

ETS capacities. To provide more insight into the changes observed in ROUTINE respiration, we measured the ETS capacity by uncoupling respiration with CCCP in permeabilized myotubes in the presence of pyruvate, glutamate, malate, and succinate (PGMS). These substrates supply electrons to the ETS via both complexes I and II. As shown in Fig. 5*A*, ETS capacity was decreased with UA ($P < 0.05$) but not with UA+MEL or MEL alone ($P > 0.05$) compared with CON. Administration of rotenone to block electron flux through complex I preserved the decrease in flux due to uric acid ($P < 0.05$ vs. CON; Fig. 5*B*). Collectively, our data suggest that *1*) the decrease in oxygen flux due to UA involves electron supply via complex II and *2*) melatonin treatment significantly counteracts the decrease in flux due to uric acid.

OXPHOS capacities with complex I and complex I+II substrates. Figure 6*A* represents the OXPHOS capacity of permeabilized C_2C_{12} myotubes supplied with PGM in the presence of 5 mM ADP. These substrates produce NADH when oxidized and therefore supply electrons to complex I only. Oxygen flux in UA and CON were similar at ~6 pmol O_2/s·ml/CIV ($P > 0.05$ vs. CON), but both MEL and UA+MEL significantly increased flux to ~15 pmol O_2/s·ml/CIV ($P < 0.05$ vs. CON). Addition of succinate (Fig. 6*B*), which supplies electrons to complex II, caused oxygen flux to increase to ~24 pmol O_2/s·ml/CIV in CON, UA+MEL, and MEL groups. In contrast, only a small increase in oxygen flux, up to ~10 pmol O_2/s·ml/CIV, was registered in the UA group. These results provide further evidence that UA reduced oxygen flux via complex II and had little or no effect on complex I-mediated electron flow.

LEAK respiration. To assess LEAK respiration in response to the treatments, 2.5 μM oligomycin was added to the oxygraph medium to inhibit ATP synthase in permeabilized myotubes supplied with PGMS and ADP. As shown in Fig. 7, there was no significant difference in LEAK respiration between CON and UA, UA+MEL, or MEL ($P > 0.05$).

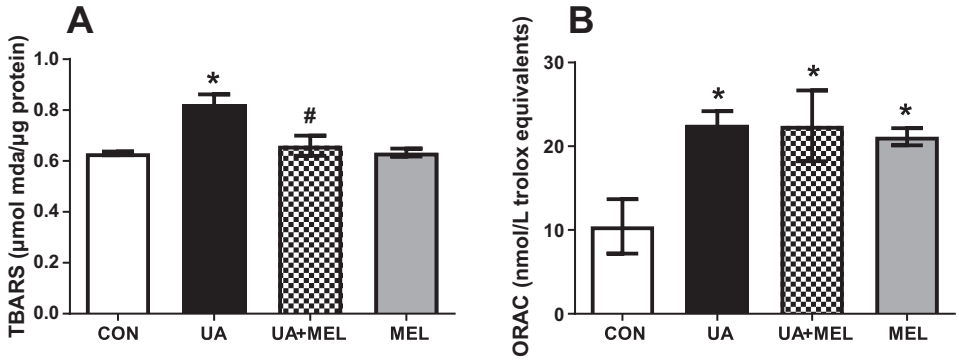

Fig. 2. *A*: TBARS results of C_2C_{12} myotubes exposed to uric acid (750 μM, 72 h) with and without melatonin (10 nM, 72 h) compared with control (CON). Melatonin treatment blocked the increased TBARS in the UA group (UA+MEL vs. UA). *B*: ORAC results of C_2C_{12} myotubes exposed to uric acid (750 μM, 72 h) with and without melatonin (10 nM, 72 h) compared with CON. Data are represented as means ± SE; $n = 6$ per group: $^{*}P < 0.05$ (vs. CON); $^{\#}P < 0.05$ (UA+MEL vs. UA).

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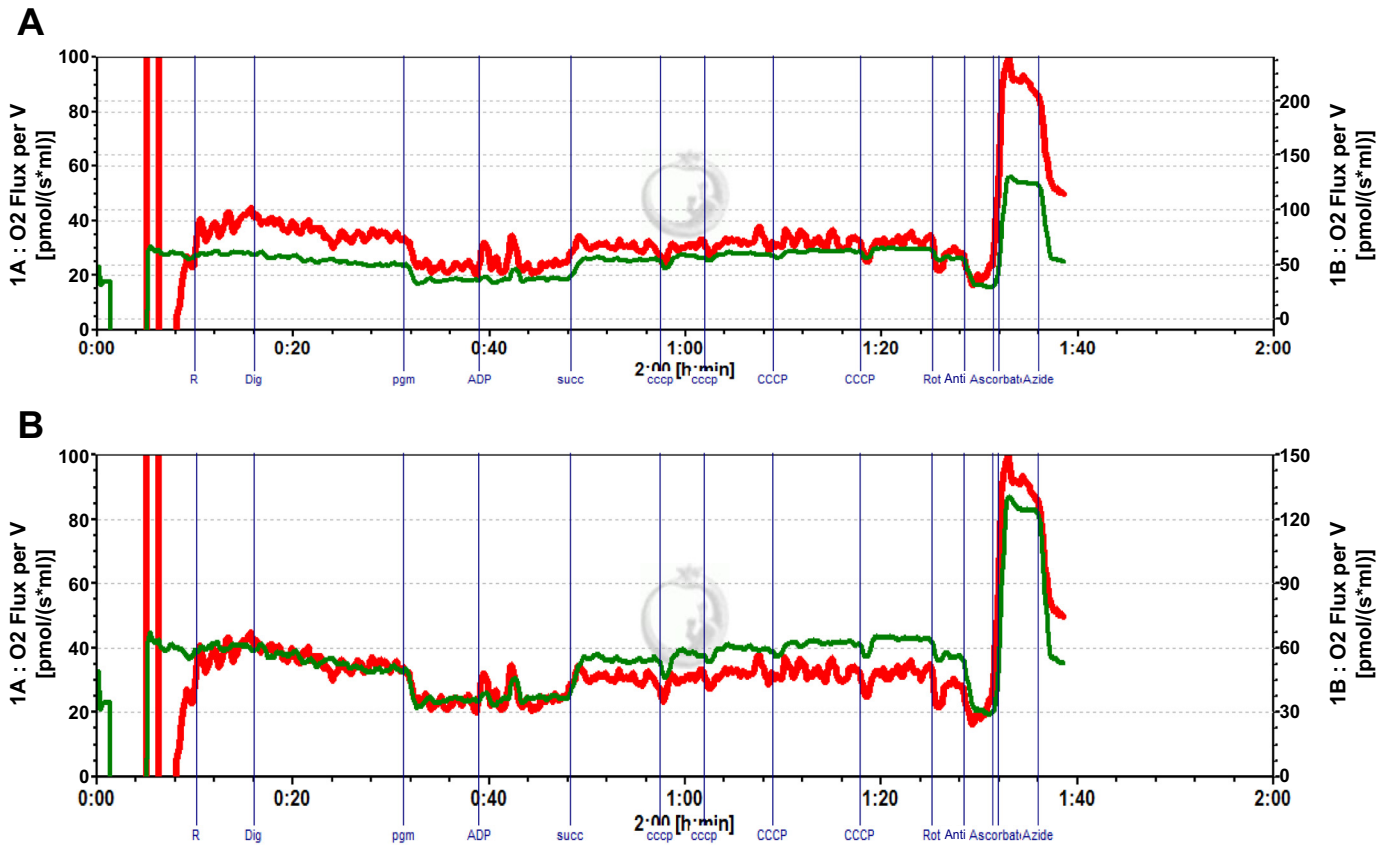

Fig. 3. Representative oxygraph traces for CON (red) and UA (green) (*A*) and for UA (red) and UA+MEL (green) (*B*). The SUIT protocol is also indicated. R, ROUTINE respiration; Dig, digitonin; pgm, pyruvate, glutamate, malate; ADP, adenosine diphosphate; SUCC, succinate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Rot, rotenone; Anti, Antimycin; Ascorbate, ascorbate/*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Azide, sodium azide.

DISCUSSION

In this study we investigated the effects of excess uric acid (750 μM, for 72 h) on differentiated C_2C_{12} myotube TG content, oxidative stress, and mitochondrial function. Our second aim was to investigate whether treatment with the powerful antioxidant melatonin (10 nM, for 72 h) (30, 31) would counteract the effects of excess uric acid. In human plasma, a uric acid concentration of 250 μmol/l is considered normouricemia and 500 μmol/l hyperuricemia; therefore the dose of uric acid chosen for our study mimics severe hyperuricemia (13). Our findings demonstrate that excess uric acid increased TG content, TBARS, and antioxidant capacity, relative to the controls. Uric acid also induced mitochondrial dysfunction by reducing ROUTINE respiration and complex II-linked OXPHOS and ETS capacities. When uric acid and melatonin at a physiological concentration of 10 nM (43) were given simultaneously, uric acid-induced oxidative stress, TG accumulation, and mitochondrial dysfunction were blocked.

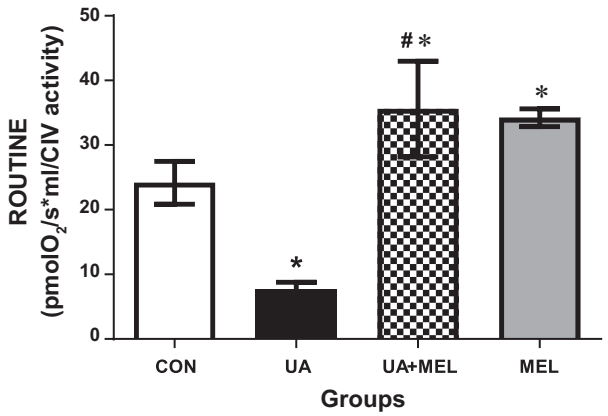

Fig. 4. ROUTINE respiration of C_2C_{12} myotubes exposed to uric acid (750 μM, 72 h) with and without melatonin (10 nM, 72 h) compared with control (CON). Melatonin treatment increased ROUTINE respiration (MEL vs. CON) and blocked the UA-induced reduction of ROUTINE respiration (UA+MEL vs. UA). Data are represented as means ± SE; $n = 6$ per group: $^*P < 0.05$ (vs. CON); $^{\#}P < 0.05$ (UA+MEL vs. UA).

Excess Uric Acid Increases Lipid Peroxidation (TBARS)

Although increased oxidative stress due to uric acid exposure has not previously been reported in C_2C_{12} myotubes, our observation is not unexpected. Previous studies have reported that uric acid caused ROS production and increased oxidative stress (35) in many cell lines including adipocytes (7, 32) and hepatocytes (13). The hydrophobic environment created by lipids inside cells is unfavorable for the antioxidant effects of uric acid but, instead, promotes its prooxidant properties (35). In this environment uric acid increases ROS production, which induces oxidative damage to LDL and membrane lipids (35). In the presence of copper ions (Cu^+ and Cu^{2+}) uric acid is known to oxidize LDL and lipid hydroperoxides (1). When it is degraded by peroxynitrite (46), uric acid produces amino-

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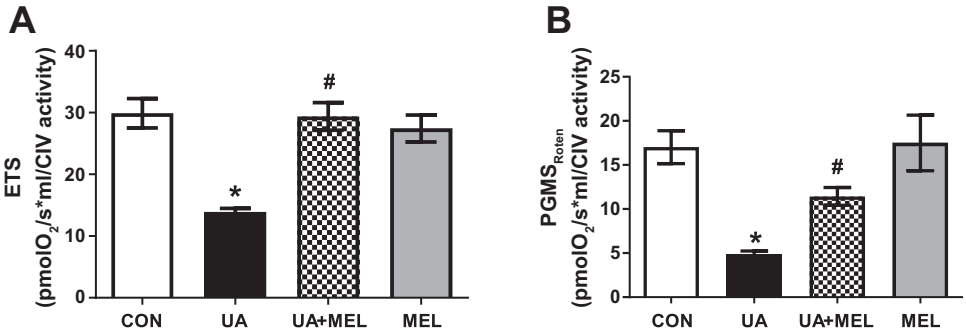

Fig. 5. ETS capacity of C_2C_{12} myotubes exposed to uric acid (750 μM, 72 h) with and without melatonin (10 nM, 72 h) compared with control (CON). Pyruvate, glutamate, malate, and succinate (PGMS) were used as substrates. Melatonin treatment blocked the UA-induced reduction of ETS capacity (UA+MEL vs. UA). *A*: oxygen flux with electron flow via complexes I and II. *B*: oxygen flux after complex I is inhibited by rotenone. Data are represented as means ± SE; $n = 6$ per group: $^{*}P < 0.05$ (UA vs. CON); $^{\#}P < 0.05$ (UA+MEL vs. UA).

carbonyl, urate anion, and urate degradation products that oxidize LDL and liposomes (19, 34).

We performed a TBARS assay to determine the peroxidation status of cells as a proxy for oxidative stress. This assay measures MDA present in the sample (11). It has been argued that this assay does not provide a precise oxidative state of cells because MDA *1*) is only one of several end products formed when certain primary and secondary lipid peroxidation products decompose, *2*) is neither the sole end product of lipid peroxide formation and decomposition nor a substance generated exclusively through lipid peroxidation, and *3*) is not generated by all lipid peroxidation (10). Future studies of the effects of uric acid on the oxidative state of muscle cells using additional markers of lipid peroxide formation and decomposition could be used to corroborate our data.

Antioxidant Capacity of Myotubes Is Increased in Response to Excess Uric Acid

We performed the ORAC assay to measure the antioxidant capacity of C_2C_{12} myotubes. This assay measures the oxidative degradation of fluorescein after being combined with the peroxyl radical generator 2,2′-azobis-(2-amidinopropane)-dihydrochloride. These radicals result in the loss of fluorescence and antioxidants within the sample and delays the decay of the fluorescent signal recorded on a fluorimeter. The assay has some limitations: *1*) it does not discriminate between the types of antioxidants in the sample, and *2*) it only measures antioxidant activity against peroxyl radicals. The assay therefore does not measure the full antioxidant capacity of the sample tested, despite being a frequently used assay.

Excess uric acid increased antioxidant capacity in our study relative to controls (Fig. 2*B*), but this is not unexpected. Mature skeletal myotubes have sophisticated enzymatic antioxidant systems, which renders them flexible in their response to changes in redox conditions (3, 28). A major class of enzymatic antioxidants, which protects against O_2^-, a primary ROS, is superoxide dismutase, which catalyzes transformation of O_2^- to H_2O_2. The H_2O_2 is subsequently converted to H_2O and O_2 by catalase or reduced to H_2O by glutathione peroxidase (28, 44). Apart from superoxide dismutase, catalase, and glutathione peroxidase, which mount a primary defense against ROS, there are also enzymes that mount a secondary phase of antioxidant defense. For example, γ-glutamylcysteine synthetase synthesizes reduced glutathione, which not only maintains vitamins E and C in their active reduced states but also reduces $HO^{\cdot}$ and O_2^- (28, 50). Another example is heme oxygenase-1, which synthesizes the reductants biliverdin and bilirubin, which protects cells from both peroxyl radicals and H_2O_2 (2, 39). Expression of superoxide dismutase, catalase, and glutathione peroxidase as well as γ-glutamylcysteine synthetase and heme oxygenase-1 may be elevated in the process of oxidative stress (12, 28, 44). Other antioxidant systems in skeletal muscle that may play a role during the process of oxidative stress include NAD(P) H:quinone acceptor oxidoreductase 1, which moderates the production of reduced semiquinones; thioredoxin, which reduces disulfide bonds; and glutaredoxin, which reduces protein thiols and peroxiredoxin systems that act as peroxidases (28). Therefore, we propose that the increased antioxidant capacity of C_2C_{12} myotubes may be an adaptive

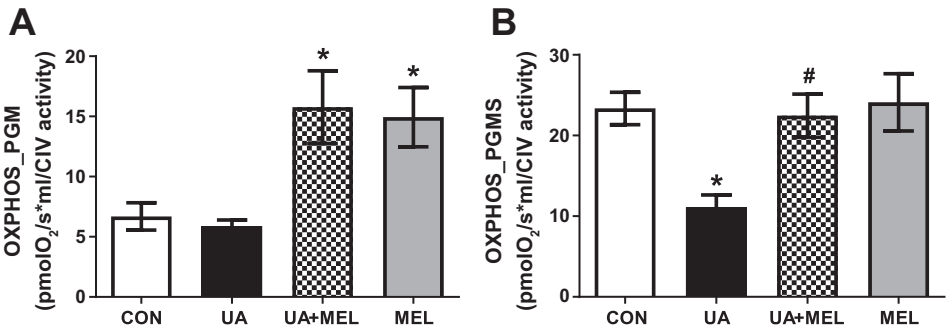

Fig. 6. OXPHOS capacity of C_2C_{12} myotubes exposed to uric acid (750 μM, 72 h) with and without melatonin (10 nM, 72 h) compared with control (CON) when pyruvate, glutamate, and malate (PGM; *A*) and pyruvate, glutamate, malate, and succinate (PGMS; *B*) are used as substrates. PGM are complex I-linked substrates, and PGMS are complex I+II-linked substrates. In *A*, melatonin treatment increased OXPHOS but UA had no effect. In *B*, UA reduced OXPHOS capacity but melatonin blocked the UA-induced reduction (UA+MEL vs. UA). Data are represented as means ± SE; $n = 6$ per group: $^{*}P < 0.05$ (vs. CON); $^{\#}P < 0.05$ (UA+MEL vs. UA).

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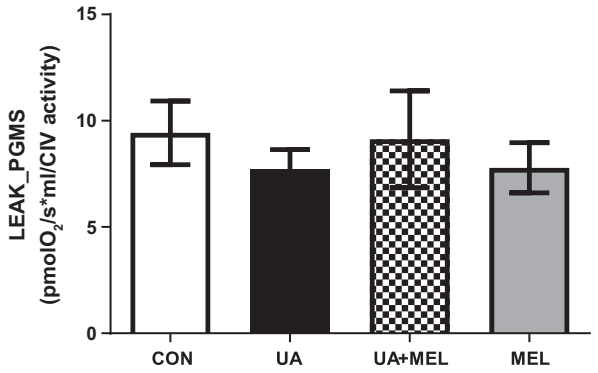

Fig. 7. LEAK respiration in the presence of ADP of C_2C_{12} myotubes exposed to uric acid (750 μM, 72 h) with and without melatonin (10 nM, 72 h) compared with control (CON). LEAK was induced by oligomycin in the presence of pyruvate, glutamate, malate, and succinate (PGMS) substrates (LEAK_PGMS). Data are represented as means ± SE; $n = 6$ per group.

response to the prooxidant actions of uric acid in the intracellular milieu.

Excess Uric Acid Causes Triglyceride Accumulation

In our study, excess uric acid also caused TG accumulation compared with controls. In line with the above hypothesis, we speculate that excess uric acid possibly increased ROS production, which may have triggered TG accumulation. Saha et al. (33) demonstrated that in the presence of glucose skeletal muscle is capable of synthesizing TGs from cytosolic citrate. Furthermore, they demonstrated that inhibition of ATP citrate lyase, the cytosolic enzyme that breaks down citrate to acetyl CoA and oxaloacetate, blocks synthesis of malonyl-CoA (the committed step in TG formation from acetyl-CoA) in soleus muscle (33). It is therefore reasonable to speculate that the TG accumulation seen in the present study was due to a similar cascade of citrate-mediated lipogenic events. However, this speculation needs empirical evaluation.

Excess Uric Acid and Insulin Resistance

The relationship between uric acid and insulin resistance was not investigated in the present study. However a strong positive relationship has previously been reported between the two variables (51). It is unlikely that the high TG levels in uric acid-treated cells in the present study play a significant role in this relationship, since numerous studies have shown that elite athletes have high intramuscular TG levels but have superior insulin sensitivity (6). Rather, the oxidative status of the cell is more likely to explain the relationship. Zhu et al. (51) induced acute hyperuricemia in mice by potassium oxonate treatment and observed increases in insulin resistance and ROS. They further showed that high uric acid inhibited phospho-Akt (Ser473) response to insulin and increased phosphor-IRS1 (Ser307) in muscle. In a separate experiment, they showed that lowering ROS levels by *N*-acetylcysteine administration improved insulin resistance and blocked high uric acid-induced IRS1 activation and Akt inhibition in HepG2 cells. Collectively, their data show that increased uric acid level directly inhibits IRS1 and Akt insulin signaling and induces insulin resistance by a mechanism that is mediated by a ROS pathway.

Excess Uric Acid Impairs Complex II-Mediated Mitochondrial Function

Our study demonstrates that excess uric acid reduced OXPHOS and ETS capacities by impairing complex II respiration. The mechanism underlying this effect is still unknown. However, it is well known that complex II is a tetrameric enzyme composed of a soluble catalytic heterodimer in the matrix and a heterodimer of membrane subunits in the inner mitochondrial membrane (8, 40, 49). As an enzyme of the TCA cycle, complex II exhibits succinate dehydrogenase activity, whereby the soluble subunits A and B in the matrix oxidize succinate to fumarate. As a component of the electron transport system, complex II also mediates succinate: ubiquinone oxidoreductase activity, whereby electrons from succinate oxidation are transferred to the quinone binding pocket at the interface of the membrane heterodimer (subunits C and D) to reduce ubiquinone (Q) to ubiquinol (QH_2). Schwall et al. (36) have shown that functional cardiolipins are essential for the structure and function of complex II because they *1*) maintain the interaction between the catalytic dimer and membrane subunits and *2*) promote electron flux from succinate to the Q reduction site in the bilayer. However, this phospholipid is particularly susceptible to ROS-induced damage, and its peroxidation has been associated with mitochondrial dysfunction in multiple tissues in several pathophysiological conditions (26). Although the effect of cardiolipin peroxidation on complex II structure and activity has not been explicitly investigated, the possibility exists that it causes the association of the soluble catalytic heterodimer with the membrane heterodimer to become unstable and promote the transfer of succinate-derived electrons to O_2 (to form O_2^-) and reduce transfer to Q. This would compromise oxygen flux through the complex and increase ROS as seen in this study. This hypothesis requires further investigation.

Melatonin Improves Uric Acid-Induced Oxidative Stress, Triglyceride Content, and Mitochondrial Function

In this study we further investigated whether treatment with melatonin would counteract the effects of excess uric acid. It has been reported that melatonin is 100 times more effective at scavenging ROS compared with vitamin E (20, 27). It exerts an antioxidant effect by directly scavenging ROS, as it reacts with the C-2 position of its indole ring (38, 41, 42). Melatonin also activates signaling pathways that induce early antioxidant gene transcription to modulate antioxidant enzyme expression (30, 31).

In our study, melatonin treatment increased antioxidant capacity in myotubes. Given its ability to induce antioxidant gene expression, this observation was expected. It also blocked uric acid-induced increases in oxidative stress, TG accumulation, and complex II dysfunction. These observations point to the therapeutic potential of melatonin in hyperuricemia-induced cellular dysfunction and confirm our hypothesis that TG and mitochondrial dysfunction seen with uric acid was a result of oxidative stress. The data also suggest that melatonin could be used as a prophylactic against excess uric acid.

We also observed that melatonin increased complex I-linked OXPHOS capacity (Fig. 6*A*). Although the underlying mechanism is not known, we ascribed it to melatonin's ability to modulate the activities/expression of enzymes associated with

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complex I-linked metabolism (30, 31). In support, Martín et al. (22) previously reported that administration of melatonin in the absence of pathology increased the activity of the complex. This property of melatonin may also explain the increase in ROUTINE respiration that we observed (Fig. 4).

Suggested Future Studies

The results of our study provide insights into the impact of excess uric acid on C_2C_{12} myotubes and justifies future studies of the functional significance of this metabolite in animal models. The increase in intramuscular TG content and the reductions in ROUTINE respiration as well as OXPHOS and ETS capacities need to be verified in these models. The prophylactic and therapeutic potential of melatonin in hyperuricemia also deserves careful analysis in live animal models. The finding that melatonin countered the effects of excess uric acid at the low concentration of 10 nM, which can be obtained from consumption of fruits, vegetables, and red wine (a Mediterranean-like diet) (9, 23, 25, 29, 47), is of practical interest and also warrants further interrogation.

GRANTS

This work was funded by National Research Foundation (NRF)-Department of Science and Technology (DST) Collaborative Postgraduate Training Grant 443739 to E. O. Ojuka.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

G.J.M., B.M.A., and D.M.B. performed experiments; G.J.M. and B.M.A. analyzed data; G.J.M. and E.O.O. prepared figures; G.J.M. and B.M.A. drafted manuscript; G.J.M., D.M.B., and E.O.O. edited and revised manuscript; G.J.M., B.M.A., D.M.B., and E.O.O. approved final version of manuscript; D.M.B. and E.O.O. interpreted results of experiments.

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