ABSTRACT: Mitochondrial cytopathies are associated with increased free radical generation and paracrystalline inclusions. Paracrystalline inclusions were serendipitously found in a young male athlete with a very high respiratory exchange ratio during steady-state exercise; he also had an unusually low aerobic capacity. Direct sequencing of the mitochondrial DNA (mtDNA) coding regions revealed a novel missense mutation (G15497A) resulting in a glycine—serine conversion at a highly conserved site in the cytochrome b gene in the subject, his mother, and sister. Cybrids, prepared by fusion of the subject’s platelets with either U87MG pH or SH-SY5Y pH cells, generated higher basal levels of reactive oxygen species (ROS), had a lower adenosine triphosphate (ATP) content, and were more sensitive to oxygen and glucose deprivation and peroxynitrite generation compared to control cybrids with wild-type mtDNA. Cell survival was significantly enhanced with 50 mmol/L creatine monohydrate (CM) administration. The subject was also treated with CM (10 g/d) for a period of 5 weeks and a repeat muscle biopsy showed no paracrystalline inclusions. The results suggest that the development of exercise-induced paracrystalline inclusions may be influenced by the G15497A mtDNA mutation, and that CM mitigates against the pathological consequences of this mutation.

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ATTENUATION OF FREE RADICAL PRODUCTION AND PARACRYSTALLINE INCLUSIONS BY CREATINE SUPPLEMENTATION IN A PATIENT WITH A NOVEL CYTOCHROME b MUTATION

MARK A. TARNOPOLSKY, MD, PhD,1 DAVID K. SIMON, MD, PhD,3 BRIAN D. ROY, PhD,1 KATHY CHORNEYKO, MD,2 STUART A. LOWTHER, MSc,1 DONALD R. JOHNS, MD,3,4 JAGDEEP K. SANDHU, PhD,5 YAN LI, MD,5 and MARIANNA SIKORSKA, PhD5

1 Department of Medicine, McMaster University Medical Center, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada
2 Department of Pathology and Molecular Medicine, McMaster University Medical Center, Hamilton, Ontario, Canada
3 Department of Neurology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA
4 Department of Ophthalmology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA
5 Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada

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Point mutations in mitochondrial DNA (mtDNA) have been associated with a variety of medical conditions.38 Exercise intolerance is a common symptom in patients with pathological mtDNA mutations.1,52,53 Mutations in the cytochrome b region of mtDNA have been reported in patients with exercise intolerance.1 There have been scattered anecdotal reports that well-trained endurance athletes with reduced exercise capacity may harbor occult mitochondrial DNA (mtDNA) mutations17,50; however, definitive evidence for mitochondrial mutations have not yet been documented in the athlete population.35,50

One consequence of mitochondrial electron transport chain dysfunction is an increase in free radical production,27,31,36 particularly with mutations affecting complex I and III.34 Mitochondrial cytopathies are also associated with a reduction in phosphocreatine

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; CK, creatine kinase; CFDA, 5-carboxyfluorescein diacetate; CM, creatine monohydrate; EDTA, ethylene-diamine tetraacetic acid; FBS, fetal bovine serum; mRNA, messenger RNA; mtDNA, mitochondrial DNA; mtCK, mitochondrial creatine kinase; OGD, oxygen and glucose deprivation; PBS, phosphate-buffered saline; PCR, phosphocreatine; PCR, polymerase chain reaction; RER, respiratory exchange ratio; RFLP, restriction fragment length polymorphism; ROS, reactive oxygen species; VO2max, maximal oxygen consumption

Key words: creatine kinase; cybrids; exercise; dietary supplements; mitochondrial myopathy

Correspondence to: M. A. Tarnopolsky; e-mail: tarnopol@mcmaster.ca

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Oxidative Stress and Creatine
stores\textsuperscript{5,23,51} and a compensatory upregulation of mitochondrial creatine kinase (mtCK).\textsuperscript{48} Paracrystalline inclusions are intramitochondrial accumulations of dimeric mitochondrial creatine kinase (mtCK) and are a histopathological feature in some mitochondrial cytopathies.\textsuperscript{30,40} The mtCK enzyme exists in both a dimeric and octameric form, with the energy shuttling function being optimized in the octameric form.\textsuperscript{42} Peroxynitrite-mediated free radical generation favors a dimeric configuration and subsequent crystallization.\textsuperscript{41} It is possible that high-intensity exercise induces paracrystalline formation consequent to increased free radical production secondary to the high mitochondrial electron flux rate,\textsuperscript{4,18,37} particularly if the athlete harbored a mtDNA polymorphism that caused an alteration in electron flux through complex I or III.\textsuperscript{34}

From a treatment perspective, a reduction in free radical generation could attenuate the generation of paracrystalline inclusions and is one of the theoretical reasons for the use of antioxidants as therapeutic agents for mitochondrial cytopathies.\textsuperscript{22} Creatine monohydrate (CM) supplementation could also be a therapeutic agent in mitochondrial cytopathies by increasing resting muscle phosphocreatine (PCr) content or resynthesis rate,\textsuperscript{5,12,16,47} stimulating mitochondrial respiration,\textsuperscript{28} or providing a direct antioxidant effect.\textsuperscript{21} There is also evidence that CM can reverse paracrystalline inclusion formation in vitro.\textsuperscript{26,29} Some,\textsuperscript{5,49} but not all,\textsuperscript{20} studies have shown that CM supplementation may enhance function in patients with mitochondrial cytopathies.

We found paracrystalline inclusions in a young male endurance athlete who also had an abnormally high respiratory exchange ratio during exercise (common in mitochondrial myopathies\textsuperscript{46,49}). Subsequently, we established that he harbored a novel mutation in the mtDNA-encoded cytochrome \textit{b} gene (G15497A). We hypothesized that the paracrystalline inclusions resulted from a pathological upregulation of mtCK and oxidative stress generated by the defective mitochondrial respiratory chain and further amplified by the intensive exercise training. Furthermore, we predicted that there would be a reduction in the paracrystalline inclusions following CM supplementation and induction of the inclusions with an increase in exercise intensity and volume. Finally, we anticipated that transmitochondrial cybrids generated from the subject’s mtDNA would demonstrate an enhanced vulnerability to peroxynitrite free radical and oxygen-glucose deprivation stress with a reduction in cellular energy charge if the mtDNA mutation was contributing to the in vivo observations.

**MATERIALS AND METHODS**

**Subjects.** The proband (Subject 1) was a 22-year-old male endurance athlete performing triathlon training for approximately 6 years who volunteered for a study looking at gender differences in intramuscular lipid. He was training for 12–16 hours per week (running, cycling, and swimming). Analysis of an electron micrograph of a needle biopsy sample of his vastus lateralis revealed abundant paracrystalline inclusions (Fig. 1). This prompted an evaluation including exercise testing, determination of muscle electron transport chain enzyme activity and adeno-

![FIGURE 1. Electron micrograph of skeletal muscle from Subject 1. (A) Pretreatment biopsy showing numerous intramitochondrial paracrystalline inclusions (original magnification ×5000). (B) Higher magnification of an area containing numerous paracrystalline inclusions (original magnification ×30,000).](image-url)
sine triphosphate (ATP), creatine and phosphocreatine content, mtDNA sequencing, western blotting for mtCK protein content, and in vitro studies of hybrid cells (see later). His past medical history and family history were unremarkable. He had one healthy sister (Subject 2) and a healthy father, but his mother (Subject 3) developed seizures in her late teens and remained on carbamazepine. The neurological examinations for all three subjects were normal as were resting plasma lactate and ammonia concentrations. Each of the three participants gave written, informed consent for all aspects of testing in accordance with the policies of the Hamilton Health Sciences Corporation Ethics Committee. Furthermore, the original and subsequent exercise studies in which the athlete participated were approved by this Ethics Committee.

**Initial Testing.** (Subject 1 unless otherwise stated.) Graded cycle ergometry exercise was performed to exhaustion to determine maximal oxygen uptake (VO$_{2\text{max}}$) using a metabolic cart, as previously described. A muscle biopsy of the vastus lateralis was taken from both Subjects 1 and 3 and sent for histochemical analysis (NADH-tetrazolium reductase, actomysin adenosine triphosphatase, modified Gomori trichrome, succinate dehydrogenase, cytochrome c oxidase, alkaline and acid phosphatase, Congo red, oil-red-O, elastic van Gieson, myoadenylate deaminase, phosphorylase, hematoxylin and eosin, and periodic acid–Schiff stains). The maximal activity of several mitochondrial electron transport enzymes (complex I + III, II + III, IV, and citrate synthase) were determined in skeletal muscle from Subject 1.

**DNA Sequencing and Related Studies.** Total cellular DNA was extracted from muscle and blood samples using a standard protocol of overnight digestion with proteinase K and sodium dodecyl sulfate, followed by extractions in phenol and chloroform, and finally ethanol precipitation. The polymerase chain reaction (PCR) amplification, sequencing protocols, and primers have been described previously. Sequencing was performed on an ABI-377 automated sequencer (Perkin-Elmer, Norwalk, CT). PCR amplification of the cytochrome b region was performed with the following primers: forward primer: 5′-CTC CGT GCT GCA ACT-3′, np 15101–15115; and reverse primer: 5′-GGC TGG CAG TAA TGT A-3′, np 16112–16097. Restriction digests were performed with AclII (New England Biolabs, Beverly, MA) and analyzed by ultraviolet illumination of a 2% agarose gel permeated with ethidium bromide. A normal control DNA sample was included in each assay to confirm complete digestion by the enzyme. We also cloned a blood-derived PCR product from Subject 3 (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) and selected 99 clones for subsequent PCR/restriction fragment length polymorphism analysis as noted earlier.

**Creatine and Exercise Intervention Studies.** Subject 1 consumed creatine monohydrate (CM; Neotine, Avicena Group, Palo Alto, CA) orally for 5 weeks (5 g twice daily), kept his training consistent, and underwent a repeat needle muscle biopsy (post-CM). Maximal and submaximal cycling exercise tests (15 min at 54% of VO$_{2\text{max}}$) were completed before and after CM supplementation with plasma lactate and ammonia, and creatine kinase values determined before and after each test. He then participated in a separate study designed to determine whether resistance exercise training would have a favorable effect on muscle function in patients with mitochondrial myopathies, in which he performed unilateral heavy-knee-extension strength training 3 days per week for 2 months at an intensity of 70% of his one-repetition maximum with three or four sets of 10 repetitions. During the duration of the resistance training program his usual endurance exercise training program was reduced in volume and intensity by about 50% (hence the contralateral leg experienced a relative reduction in exercise). After the 2-month training session, he had needle muscle biopsies taken from the vastus lateralis muscle of each leg.

Each of the biopsies was cut into ~1-mm$^3$ portions and immediately fixed in precooled 2% glutaraldehyde in 0.1% sodium cacodylate buffer and postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, and infiltrated with graded mixtures of propylene oxide and Spurr’s resin. Semithin, toluidine blue-stained sections were examined to assess the orientation and quality of the tissue sections. Thin sections were cut and stained with uranylacetate and lead citrate. Images were obtained using a transmission electron microscope (1200×, Japanese Electron Optics, Ltd., Tokyo, Japan) and representative photomicrographs were obtained. For each of the four specimens (initial biopsy, post-CM, increased exercise, and decreased exercise leg), a minimum of 10 muscle fibers and 650 mitochondria were reviewed blindly by a single evaluator and the number of muscle mitochondria containing a paracrystalline inclusion, electron density, or early paracrystalline inclusion were counted (test–retest variability was >0.95).

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In addition to electron microscopy, western blotting was completed for mtCK content as previously described. In addition, ATP, creatine, and PCr concentrations were determined from lyophilized muscle samples taken at baseline and post-CM supplementation using methods described elsewhere.

Generation of Cybrids by Platelet Transformation of ρ° Cells. Cybrids were generated on a background of two human cell lines, U87MG glioblastoma (ATCC HTB-14) and SH-SY5Y neuroblastoma (ATCC CL-2266), devoid of their own mtDNA (ρ° cells). Parental U-87MG and SH-SY5Y cells were grown in Eagle’s minimum essential medium (Gibco BRL, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and 1× antibiotic–antimycotic solution (Gibco BRL) at 37°C in 5% CO2. The U-87MG and SH-SY5Y ρ° cells were generated by culturing cells for 3 months in the presence of 0.25 μg/ml and 1.5 μg/ml ethidium bromide, respectively, in complete minimal essential medium containing 100 μg/ml pyruvate, 50 μg/ml uridine, and 4500 mg/L glucose (ρ° medium). Cells were replated once per week and the medium was changed every 2–3 days. The absence of mitochondrial DNA was determined by PCR using D-loop–specific primers (forward primer: 5’-CCC TCC CAC TCC CAT ACT CCC-3’, np 16440–16459; reverse primer: 5’-ACG GGC GTG TAC GCC G-3’, np 914–897; Genbank D38112.1).

The ρ° cells were transformed with platelets obtained from Subject 1 (JL cybrids) and from two different control individuals, a 38-year-old male endurance athlete with a training background similar to Subject 1 (MT cybrids) and a 45-year-old healthy and physically fit man (MK cybrids), using a previously described protocol. Briefly, platelets were isolated from 10-ml samples of fresh blood drawn into an evacuated heparinized tube and mixed with a suspension of ρ° cells (0.8–1.0×10^6 cells in 2 ml of calcium-free Dulbecco’s modified Eagle’s medium). Cellular fusion was facilitated by an addition of polyethylene glycol 1500 solution. The cell/platelet mixtures were then diluted to a final volume of 10 ml with the ρ° cell medium and plated into 96-well plates by a serial dilution. The cells were allowed to recover for 1 week in the ρ° medium, and cybrids containing exogenous platelet mitochondria were selected by culturing in minimal essential medium (lacking pyruvate and uridine) supplemented with 10% dialyzed heat-inactivated FBS. Surviving cybrid clones were expanded and analyzed for the presence of mtDNA mutation by PCR (forward primer: 5’-CCT CCT GCT TGC AAC TAT AG-3’, np 14514–14533; reverse primer: 5’-CAT GGT GGC TGG CAG TAA TG-3’, np 15532–15513) and restriction digestion with AvrII, as described earlier.

Measurement of Cellular ATP. The ATP assay was performed using the Quantitative ATP Monitoring Kit (Thermo Labsystems, Helsinki, Finland) according to the manufacturer’s protocol. Briefly, 5×10^4 cells were plated in T25 flasks and then harvested for the assay after 7 days when 80% confluent. The cells were harvested in a buffer containing 20 mmol/L glycine, 50 mmol/L MgSO4, and 4 mmol/L ethylenediamine tetraacetic acid (EDTA; pH 7.4), and the assay was carried out with 20 μl of the sample mixed with 2–5 μl of luciferase–luciferin solution. Intensity of the emitted light was measured using a Model 1450 Microbeta luminescence counter and the amount of ATP produced was determined from the standard curve constructed using 10–100 pmol of ATP. Protein concentration was measured using Bradford reagent (Bio-Rad Laboratories, Hercules, CA).

Measurements of Reactive Oxygen Species (ROS). ROS production was assessed using flow cytometry following cell staining with CMH2DCFDA [5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate, aceylester]. Cells were harvested by trypsinization, washed, and resuspended in phosphate-buffered saline containing 1% bovine serum albumin. The cells (1×10^6 cells/0.5 ml) were stained for 20 min at 37°C with 5 μmol/L CMH2DCFDA (Molecular Probes, Eugene, OR). CMH2DCFDA was excited at 488 nm and the emitted fluorescence was analyzed at 525 nm using a flow cytometer and EXPO32 software (Applied Cytometry Systems, Sheffield, UK).

Cybrid Exposure to Metabolic and Oxidative Stress. Cybrids were plated in 24-well plates (3×10^4 cells/well) and 2 days later placed in glucose- and serum-free medium and incubated for 24 h at 37°C in a Gas Pak 100 chamber containing Gas Pak Plus gas generator envelopes (VWR International, Mississauga, ON, Canada). The generator envelopes catalytically reduce the oxygen concentration while providing a humidified atmosphere with 5% CO2 [oxygen and glucose deprivation (OGD)]. In some experiments, the cybrids were placed for up to 48 h in glucose-free medium containing 1.0 mmol/L of 3-morpholinopyrrolidine (SIN-1; Toronto Research Chemicals, North York, ON, Canada) to generate peroxynitrite. Creatine monohydrate (CM; 50 mmol/L final concentrations) was added to the media once just before
each of the treatments. Cell viability was measured immediately after completion of the treatment.

**Cell Viability Assay.** The 5-carboxyfluorescein diacetate (CFDA) assay was used to assess cell viability. Briefly, the cells were washed with phosphate-buffered saline and incubated at 37°C for 30 min with 2.5 μg/ml of 5-CFDA (Sigma) in Earle’s balanced salt solution (Sigma). Fluorescence was quantified using a CytoFluor 2300/2350 fluorescence measurement system (Millipore, Bedford, MA) with a 480/20 excitation filter and 530/25 emission filter.33 Cell viability in SH-SY5Y cells was assessed by trypan blue exclusion; blue cells were scored as dead and transparent cells were scored as live.

**Statistical Analysis.** Results were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey’s multiple comparison test or unpaired t-test where applicable. P < 0.05 was considered statistically significant. We also compared the exercise data between trials using paired t-tests, with P < 0.05 considered statistically significant. Statistical tests were performed using GraphPad Prism version 3.02 (GraphPad, San Diego, CA).

**RESULTS**

**Initial Exercise Testing.** Aerobic capacity (VO2max; 52 ml/kg per minute) for Subject 1 was lower (>2 SD) than expected for the duration and volume of exercise training (normal = 65.5 ± 5.4 ml/kg per minute, N = 12) and lower than the athlete (76 ml/kg per minute) from whom the MT cybrids were made for comparison with Subject 1. During submaximal endurance exercise for 90 min at 60% VO2max the respiratory exchange ratio (RER = VCO2/VO2) for Subject 1 was very high at 1.02 (>3 SD; normal 0.93 ± 0.02, N = 12).

**Initial Muscle Biopsy Findings.** Light microscopy revealed a prominence of subsarcolemmal staining intensity on cytochrome oxidase and Gomori trichrome stains only for Subject 1, with no evidence of cytochrome c oxidase–negative or classic ragged-red fibers for either Subject 1 or 3. Electron microscopy showed an increase in mitochondrial number, mild pleomorphism with an abundance of paracrystalline inclusions in Subject 1 (Fig. 1), and slight pleomorphism in his mother (Subject 3; not shown). The maximal activities of several electron transport chain enzymes (complex I + III, II + III, and IV) were within normal limits except for citrate synthase activity, which was above the normal range (all electron transport chain enzymes were normal when expressed relative to citrate synthase) (data not shown). Total creatine and ATP content were lower than expected compared with age- and gender-matched control values (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Muscle biochemistry of subject 1.</th>
</tr>
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<tbody>
<tr>
<td><strong>Creatine and high-energy phosphate compounds (mmol/kg dry weight)</strong></td>
</tr>
<tr>
<td>Pre-CM</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Phosphocreatine</td>
</tr>
<tr>
<td>Creatine</td>
</tr>
<tr>
<td>Total creatine</td>
</tr>
<tr>
<td>ATP</td>
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</tbody>
</table>

Pre-CM, value before creatine supplementation; post-CM, value after creatine supplementation; ATP, adenosine triphosphate.

**Paracrystalline Inclusion Response to Creatine Monohydrate and Increased Exercise Training.** Five of 12 muscle fibers contained abundant paracrystalline inclusions in the initial biopsy (Fig. 1A, Table 2). Electron micrographs from 16 other elite endurance athletes and 13 men and women before and after a strenuous 31-day endurance exercise program were reviewed and no paracrystalline inclusions were identified (minimum of 600 mitochondria evaluated per subject). Following 5 weeks of CM treatment, no paracrystalline inclusions were found and only a single electron density was seen among a total of 10 muscle fibers screened. After 2 months of increased training in one leg, Subject 1 developed multiple...
have a paracrystalline lattice-like appearance but lack the distinct regular periodicity of classical paracrystalline inclusions.

The leg that only performed moderate endurance exercise training. Densities refer to spheroid or circular electron densities. Preinclusions refer to inclusions that have a paracrystalline lattice-like appearance but lack the distinct regular periodicity of classical paracrystalline inclusions.

Early paracrystalline inclusions in 3 of 10 muscle fibers, whereas the contralateral leg showed only a single electron density and a single early paracrystalline inclusion in 10 muscle fibers. The CM supplementation intervention increased total muscle creatine by 11% and ATP content by 8% (Table 1).

The mtCK protein content in the initial muscle biopsy for Subject 1 was twofold greater as compared with both his mother (Subject 3) and an age-matched healthy male control, and was nearly identical to the mtCK content in muscle from a patient with the MELAS T3271C mutation whose sample was run simultaneously. There was no change in mtCK protein content after the 5 weeks of CM supplementation, and the increased exercise training did not alter mtCK content (data not shown).

Submaximal exercise testing before and after CM supplementation did not change basal or postexercise lactate concentration (Table 2). There were no differences in VO\textsubscript{2max} minute ventilation, oxygen consumption, or heart rate, whereas the RER was significantly lower (P<0.01) after the CM supplementation trial (Table 2).

**Sequencing and Restriction Digest Results.** In Subject 1, all coding regions of the mitochondrial genome were sequenced except for a small region of the cytochrome c oxidase I gene. We found several missense mutations that were not previously identified as polymorphisms: G15497A (cyt b) G > S; A14582G (ND6) V > A; C3992T (ND1) T > M; and A4024G (ND1) T > A. There were also two tRNA basepair (bp) substitutions that were not previously identified as polymorphisms: T7581C (Asp) and G15930A (Thr). A C311T mutation, which is not a known polymorphism, occurred in a highly variable region of the noncoding D-loop. Several known polymorphisms also were identified: CC insertion at 303, G9123A, and G14365T. In addition, there were numerous errors/consensus changes in the Cambridge sequence.2 A14582G, C3992T, and A4024G alter amino acids that are nonconserved across mammals. The T7581C mutation occurs at a nonconserved site (e.g., normally a C at this site in cows). The G15930A mutation alters a nonconserved site (e.g., normally an A at this site in mouse and rat). In contrast, the G15497A mutation converts a highly conserved glycine to a serine [glycine in each of 11 mammals analyzed, as well as in alligator (Alligator mississippiensis), chicken (Gallus gallus), carp (Cyprinus carpio), frog (Xenopus laevis), and fly (Drosophila yakuba)]. The normal amino acid is a threonine in turtle (Pelomedusa subrufa) and asparagus in sea urchin (Strongylocentrotus purpuratus), snake (Dinodon semifasciatus), and starfish (Asterina pectinifera). This mutation was not found in 100 control samples. It also is not listed in the extensive MITOMAP database of known polymorphisms (http://www.gen.emory.edu/mitomap.html), was not identified in a recently published screen of the mtDNA coding regions of 560 subjects,15 and has not previously been reported in the literature, at least to our knowledge.

The presence of the G15497A mutation was confirmed in blood from all three subjects and in muscle from Subjects 1 and 3 using restriction digestion of PCR-generated fragments. The restriction enzyme AvaII normally cuts the PCR product at two sites (np 15370 and 15493) yielding three bands (619, 270, and 123 bp). In the presence of the G15497A mutation, the AvaII enzyme cuts at one site and yields only two bands (742, 269). We found evidence for the G15497 mutation in muscle or blood DNA from each family member studied. The absence of a 619-bp band from any of the family members suggests that the mutation was homoplasmic. A faint band at approximately 120–130 bp also was visible for each family member, and was presumably an artifact based on the lack of any 619-bp band. Cloning of blood-derived DNA from Subject 3 followed by a postcloning PCR reaction and AvaII digestion for 99 clones confirmed that the G15497A mutation was present in every clone, indicating that normal mtDNA is either absent or extremely rare.

**Cybrid Analysis.** In order to eliminate effects of cellular background on cybrid behavior, the cybrids

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of mitochondria</th>
<th>No. of muscle fibers</th>
<th>Paracrystalline inclusions</th>
<th>Densities</th>
<th>Preinclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1374</td>
<td>12</td>
<td>49</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Postcreatine</td>
<td>842</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Increased training</td>
<td>925</td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>Decreased training</td>
<td>651</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Increased training refers to the leg that performed unilateral heavy strength exercise in addition to moderate endurance exercise. Decreased training refers to the leg that only performed moderate endurance exercise training. Densities refer to spheroid or circular electron densities. Preinclusions refer to inclusions that have a paracrystalline lattice-like appearance but lack the distinct regular periodicity of classical paracrystalline inclusions.
were generated using two different human cell lines: U87MG glioblastoma and SH-SY5Y neuroblastoma. For each cell line and each mtDNA donor, several cybrid clones were randomly selected and analyzed and the presence or absence of cytochrome b mutation was confirmed by the restriction analysis of PCR-amplified mtDNA fragment with AvrII (Fig. 2). All cybrids obtained from platelets of control subjects (MK and MT cybrids) showed three restriction fragments, 625, 271, and 123 bp (lanes 2, 5, and 6), characteristic of donor wild-type mtDNA (lane 1), and all cybrid clones generated by the fusion of (JL cybrids) platelets of Subject 1 produced only two fragments, 748 and 271 bp (lanes 4, 7, and 8), consistent with the donor G15497A mutation and its homoplasmic nature (lane 3). These cell transformatants were stable, and no revertants to the wild-type mtDNA were observed during multiple passages in culture. Subsequently, several cybrid clones were selected and analyzed further.

First, the cybrids were analyzed for the basal level of ROS production using flow cytometry (Fig. 3A and B). We detected a marked shift in relative fluorescence intensity of the CMH2DCFDA dye between the U87MG wild-type and mutant cybrids (from X-mean value of 1.4 to 7.1 in MK-1 vs. JL-91 and from X-mean value of 2.9 to 7.1 in MK-4 vs. JL-78), indicative of a higher ROS level in both randomly selected mutant clones. Similar differences were observed between the mutants and wild-type cybrids from a third donor (data not shown), indicating that the mutation likely affected cellular metabolism.

Second, we measured cellular ATP content and established that the steady-state ATP levels were consistently lower in the G15497A mutant clones than in the wild-type controls (Fig. 3C and D). This was true regardless of the cellular background, U87MG (Fig. 3C) and SH-SY5Y (Fig. 3D), or the donor of wild-type mtDNA (MK-1 and MT-1). Thus, the ATP content was approximately 40% lower in JL-1 mutant than in MT-1 control \((P < 0.0001, \text{Fig. 3D})\) and it was also 20% lower in the JL-91 mutant vs. MK-1 control \((P = 0.025, \text{Fig. 3C})\).

Third, the cellular responses to energy charge reduction and oxidative stress (an in vitro form of exercise stress) was also tested (Fig. 4). The U87MG cybrids were subjected to a 24-h period of OGD in the presence or absence of 50 mmol/L CM (Fig. 4A). Based on the CFDA assay, there was a significant loss of cellular viability in response to treatment in both cybrid clones \((P < 0.01 \text{ for control MK-1 cybrid and } P < 0.001 \text{ for mutant JL-91 cybrid; Fig. 4A})\) and the JL-91 cybrid was more sensitive to the treatment.

FIGURE 2. PCR amplification and restriction analysis of mtDNA. Lane 1, wild-type mtDNA from a 38-year-old MT donor; lane 2, control SH-SY5Y clone with wild-type mtDNA from the MT donor (MT-1 cybrid); lane 3, mutant mtDNA from Subject 1 (JL donor); lane 4, mutant SH-SY5Y clone carrying mtDNA of the JL donor (JL-1 cybrid); lanes 5 and 6, control U87MG cybrids MK-1 and MK-4, respectively, both with the wild-type mtDNA from a 45-year-old donor; lanes 7 and 8, mutant U87MG cybrids JL-78 and JL-91, respectively, both with the mutant mtDNA from the JL donor; lane M, 1 kilobase-plus marker.

FIGURE 3. Reactive oxygen species and ATP levels in cybrids. (A) Filled histogram, ROS level in MK-1 wild-type cybrid (X-mean value 1.4); unfilled histogram: increased ROS level in JL-91 mutant cybrid (X-mean value 7.1). (B) Filled histogram, ROS level in MK-4 wild-type cybrid (X-mean value 2.9); unfilled histogram, increased ROS level in JL-78 mutant cybrid (X-mean value 7.1). (C) Basal ATP level in U87MG cybrids (filled bar, MK-1 wild-type cybrid; open bar, JL-91 mutant cybrid). (D) Basal ATP levels in SH-SY5Y cybrids (filled bar, MT-1 wild-type cybrid; open bar, JL-1 mutant cybrid) \(* P = 0.025; *** P < 0.0001\).
The presence of 50 mmol/L CM during the OGD treatment preserved cell viability and protected both clones from cell death. Similar results were obtained with U87MG cybrids exposed for 24 h to a combination of glucose deprivation and SIN-1 (GD + SIN-1; Fig. 4B). The G15497A mutant clones (JL-1) showed a significant loss of viability (P < 0.001). Again, the addition of 50 mmol/L CM protected the cybrids from cell death (Fig. 4B). The SH-SY5Y neuroblastoma cybrids were also subjected to the same GD + SIN-1 stress (Fig. 4C). These cybrids were more refractive to the treatment than U87MG clones with no significant loss of viability at 24 h, except in the JL-1 mutant (P < 0.01; Fig. 4C), but cell death was evident in both mutant and wild-type clones after the 48-h treatment (P < 0.001). Between-subject analysis revealed that the mutant JL-1 cybrids displayed higher vulnerability to the stress than the wild-type MT-1 cybrids (P < 0.05). Overall, the presence of this mtDNA G15497A mutation was associated with a diminished ability to cope with both OGD and GD + SIN-1 stressors, regardless of the cellular background.

**DISCUSSION**

We have shown the reversal of paracrystalline inclusions in skeletal muscle with creatine monohydrate supplementation in humans. To our knowledge, this is the first report of paracrystalline reversal following any intervention in humans. These results, in combination with the in vitro data, suggest that CM has cellular effects that can confer therapeutic efficacy in patients with mitochondrial dysfunction. The current study also implicates the homoplasmic G15497A mutation with paracrystalline formation, a reduction in cellular energy charge, and increased generation of free radicals. Finally, this study adds important information on the interactions between exercise and mitochondrial structure and function.

Paracrystalline inclusions, a reduction in aerobic respiration, and an increase in mtCK activity can be induced in rat soleus muscle by feeding β-guanidinopropionic acid (β-GPA), which depletes phosphocreatine stores. Skeletal muscle from patients with mitochondrial cytopathies also show increased mtCK mRNA and mtCK protein as well as reduced phosphocreatine concentration. Mutations in mtDNA are known to increase free radical production in animals and humans, and apoptosis of myonuclei has been seen in patients with mitochondrial myopathies. Free radical donors such as peroxynitrite can destabilize the octameric structure of mtCK and result in dimer formation, which in turn can open the mitochondrial permeability transition pore. Our data show that a homoplasmic G15497A mutation is associated with abundant paracrystalline inclusions in skeletal muscle when combined with exercise, and that a reduction in cellular energy charge and increased free radical production occurred in transmitochondrial cybrids with mtDNA from Subject 1. The finding of paracrystalline inclusions in skeletal muscle from the athlete (Subject 1), but not in his mother (Subject 3), sug-
gests that the paracrystalline formation was due to an interaction between the mutation and exercise training. This finding was similar to the observation that environmental and genetic factors influence the pathological expression of other homoplastic mtDNA mutations such as the G11778A mutation that results in Leber’s hereditary optic neuropathy.\(^7\) In addition to findings showing a lack of paracrystalline inclusions in skeletal muscle from the other 16 athletes who were part of the original lipid study, we also examined electron micrographs over the past decade from over 75 men and women who were well-trained endurance and strength athletes under 35 years of age, and did not detect a single paracrystalline inclusion (Tarnopolsky et al., unpublished observations), nor have these inclusions ever been reported in the literature in well-trained athletes. These data further suggest that paracrystalline inclusions seen in Subject 1 were not due to exercise training per se.

By controlling for environmental and nuclear genetic factors, the cybrid data also suggest that the G15497A mutation resulted in a reduction in cellular energy charge and an increase in free radical generation. With long-term endurance exercise training, the subject with the G15497A mutation developed abundant paracrystalline inclusions, had a relatively low \(V_{O_2max}\), and an abnormal exercise RER response. Finally, the current data are concordant with the observation made by others that mutations in the cytochrome \(b\) region are present in people with exercise intolerance as their predominant presenting feature.\(^1\) There exists some controversy regarding the semantics of labeling a sequence alteration as “pathogenic” as compared with a polymorphism. The fact that the G15497A mutation alters a highly conserved amino acid, that mutations in this region have been reported by other investigators to cause exercise intolerance,\(^1\) that it reduces cellular ATP content when expressed in vitro, and that paracrystalline inclusions have never before been reported in the skeletal muscle of athletes all suggest pathogenic potential. The homoplastic nature of the mutation and the fact that we did not find paracrystalline inclusions in the athlete’s mother who harbored the same mutation argue that perhaps this is a polymorphism. We suggest that G15497A represents a “conditionally pathogenic” mutation and requires the superimposition of exercise in order to manifest the in vivo pathological consequences (paracrystalline inclusions).

Cardiomyocytes isolated from \(\beta\)-GPA-treated rats showed abnormal mitochondrial morphology and paracrystalline inclusions, which reversed when the medium was enriched with CM.\(^10\) The current data show that CM supplementation reversed skeletal muscle paracrystalline inclusions and that CM also prevented death of cybrid cells harboring the G15497A mutation that were exposed to cellular stress. Creatine supplementation has been shown to reduce oxidative stress in animal models of neurodegenerative disease\(^10\) and to inhibit opening of the mitochondrial permeability transition pore and inhibit apoptosis.\(^45\) Our data indicate that CM prevented cybrid cell death due to combined oxygen and glucose deprivation and peroxynitrite-mediated free radical generation. Although the exact role of CM supplementation on the reversal of paracrystalline inclusions in vivo is not fully elucidated, our data show that this was not due to a downregulation of mtCK protein content. The increase in muscle total creatine content following CM supplementation may have functioned as an antioxidant.\(^21\) Furthermore, an increase in muscle total creatine would also be expected to increase the mtCK octameric:dimeric ratio, especially with the evidence for an increase in energy charge (ATP) observed after CM supplementation.\(^41\)

The functional consequences of a reduction in paracrystalline inclusions in patients with mitochondrial myopathies are not clear. The subject in the current study did not feel subjective improvements during the 5-week CM supplementation trial and there was no effect on his maximal oxygen consumption. This finding is in contrast to animal data showing improved mitochondrial capacity in vitro after CM supplementation,\(^32\) but is consistent with human studies in mitochondrial cytopathies.\(^49\) There was, however, a reduction in the RER during submaximal exercise after CM supplementation, and RER was one of the variables that was most abnormal and consistent with the pattern seen in patients with severe mitochondrial cytopathies with paracrystalline inclusions.\(^49\) In the latter study, we did not find a change in maximal oxygen uptake following 5 weeks of CM supplementation in seven patients with severe mitochondrial myopathies, although there were improvements in muscle function.\(^49\) It is possible that a subtle effect of CM on maximal aerobic capacity would not be detected with exercise testing in studies with small numbers of patients, or that a longer treatment period is required to quantify an effect. The current data also suggest that studies evaluating the potential efficacy of treatments for mitochondrial cytopathies should include the use of measures of mitochondrial dysfunction, including oxidative stress and histological measures such as paracrystalline number.
It could be argued that the current study provides evidence that exercise induces mitochondrial dysfunction and offers some support for anecdotal reports of some athletes having significant mitochondrial alterations. Our data imply that an underlying inherited mtDNA sequence alteration was “unmasked” by excessive endurance exercise training (over 12 h/week for many years). Modest endurance exercise training actually appears to improve mitochondrial function and exercise capacity in patients with mtDNA mutations.

In summary, our study has demonstrated that paracrystalline inclusions, observed in association with a G15497A cytochrome b mutation, were dynamic, increasing with excessive exercise and decreasing with CM supplementation. Expression of mtDNA harboring this G15497A mutation in cybrid cell lines was associated with increased ROS production and reduced cellular energy charge and rendered cells more vulnerable to further insults. Under physiological stress, specifically excessive exercise/endurance training (where glucose and oxygen deprivation and oxidative stress occur), this could lead to paracrystalline inclusion formation.

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REFERENCES


