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Myoadenylate deaminase deficiency does not affect muscle anaplerosis during exhaustive exercise in humans

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1. Myoadenylate deaminase (AMPD) deficiency is present in 1–2% of the population. In theory, this deficiency may alter exercise energy metabolism by impairing the purine nucleotide cycle (PNC) and reducing tricarboxylic acid (TCA) cycle anaplerosis. The role of the PNC in TCA cycle anaplerosis is still a debated issue in physiology. Using patients with the *AMPD1* mutation will allow a human 'knockout' approach to answering this question.

2. Muscle AMPD activity and genotype (whole blood *AMPD1* analysis) was used to classify participants into three groups: $n = 3$ with absence of AMPD activity and −/− *AMPD1* genotype (homozygous); $n = 4$ with less than 50% normal AMPD activity and +/− genotype (heterozygous) and $n = 12$ with normal AMPD activity and +/+ genotype (control). Biopsies were taken from the vastus lateralis muscle before and after incremental cycle ergometry exercise to exhaustion. The muscle biopsies were analysed for AMPD activity, purine nucleotides/nucleosides and bases, creatine, phosphocreatine, amino acids, and the TCA cycle intermediates malate, citrate and fumarate.

3. Time to exhaustion on the cycle ergometer was not different between groups. Muscle adenosine monophosphate increased significantly with exercise for homozygous subjects as compared with the other groups ($P < 0.05$). Inosine monophosphate increased significantly after exercise for control ($P < 0.05$) but not for the homozygous subjects. There were no other between-group differences for any other measured variables.

4. In summary, complete and partial muscle AMPD deficiency did not affect TCA cycle anaplerosis, phosphocreatine hydrolysis, energy charge or exercise performance.

The most common known genetic abnormality of human skeletal muscle is myoadenylate deaminase (AMPD) deficiency (Norman *et al.* 1995, 1998). Myoadenylate deaminase catalyses the reaction from adenosine monophosphate (AMP) to inosine monophosphate (IMP) plus ammonia (NH_3). A deficiency of myoadenylate deaminase activity was first characterized by Fishbein *et al.* (1978). AMPD deficiency is an autosomal recessive and has been reported in about 1–2% of the population (Norman *et al.* 1995, 1998; Verzijl *et al.* 1998) with an allelic frequency of about 13% in Caucasians and African–Americans (Morisaki *et al.* 1992; Norman *et al.* 1998; Verzijl *et al.* 1998). The gene encoding the AMPD protein is termed *AMPD1* and is found on chromosome 1 (Morisaki *et al.* 1992; Sabina *et al.* 1992). Most patients with primary *AMPD1* deficiency have a C→T transition mutation at nucleotide 34 in exon 2 that results in a nonsense mutation at codon 12 and a truncated protein (Morisaki *et al.* 1992). The diagnosis has been traditionally established using the forearm ischaemic test to demonstrate no ammonia rise with a normal lactate rise (an increase of NH_3:lactate < 0.4) and by absent histochemical staining for AMPD on a frozen section of skeletal muscle (Shumate *et al.* 1979; Fishbein *et al.* 1990).

The diagnosis of *AMPD1* deficiency is complicated by the fact that the clinical phenotype can vary and there are several conditions that can mimic some of the investigative abnormalities described above. For example, in addition to primary *AMPD1* deficiency, there have been two other forms of apparent reductions in AMPD1 activity termed 'coincidental' and 'acquired' AMPD deficiency (Fishbein, 1999). In the coincidental form, AMPD deficiency is thought to co-exist with another neuromuscular disease

due to a statistical probability with a prevalent disorder. The acquired form most probably represents the co-existence of a partial reduction in AMPD activity in a heterozygous carrier with a superimposed further reduction from a primary neuromuscular disease (Fishbein *et al.* 1978, 1999). Fishbein has also described several patients who demonstrated a normal work-up for neuromuscular disease and had normal AMPD activity in skeletal muscle, yet had no NH_3 rise in response to forearm ischaemic testing (Fishbein *et al.* 1990). It was postulated that these 'functional' AMPD-deficient patients could have an impaired activation of a mutant enzyme *in vivo* (K_m mutant) or possibly an impairment of NH_3 efflux from the muscle (Fishbein *et al.* 1990). The existence of the different forms of AMPD deficiency has probably contributed to some of the discrepancies in the assessed frequency of the disorder in different patient populations.

There is an ongoing debate as to the potential significance of the primary *AMPD1* mutation to skeletal muscle metabolism (Fishbein *et al.* 1978; Sabina *et al.* 1980; Norman *et al.* 1995; Verzijl *et al.* 1998; Fishbein, 1999). For example, some have found the absence of AMPD activity (Norman *et al.* 1995) and the presence of the *AMPD1* mutation (Norman *et al.* 1998; Verzijl *et al.* 1998) in about 2% of asymptomatic adults. On the other hand, the absence of AMPD activity has been found in 8% of patients with complaints of myalgia (Kelemen *et al.* 1982). At the biochemical level, one report found a marked reduction in total purine nucleotides after exercise in a patient with AMPD enzyme deficiency (Sabina *et al.* 1980), whereas another study found that lactate accumulation, phosphocreatine hydrolysis and cellular energy charge were not differentially affected by ischaemic, isometric exercise in AMPD deficient patients as compared with controls (Sinkeler *et al.* 1987).

It is possible that the muscle fatigue and weakness reported by some AMPD deficient patients could occur via inhibition of the potential anaplerotic function of the purine nucleotide cycle (PNC) (Sabina *et al.* 1980; Flanagan *et al.* 1986; Gibala *et al.* 1997); a decrease in phosphorylation potential (Sahlin & Broberg, 1990); an increase in adenosine concentration (Blazev & Lamb, 1999); and/or acceleration of phosphocreatine hydrolysis and glycogenolysis/glycolysis to maintain phosphorylation potential (anaerobic ADP rephosphorylation).

Given the importance of TCA cycle anaplerosis to energy production in skeletal muscle during exercise (Gibala *et al.* 1997*a*), this pathway requires careful evaluation in AMPD patients. An ongoing issue in the study of cycle anaplerosis is the source of the TCA cycle intermediates, but recent evidence has questioned the role of the PNC in this process (Gibala *et al.* 1997*b*). The study of anaplerosis in patients with high grade AMPD deficiency provides a unique opportunity to determine whether or not PNC function is required for TCA cycle anaplerosis during exercise in humans. In a review of the potential role of AMPD1 in skeletal muscle, it was concluded that, 'Further study is needed to explain the significance of purine nucleotide catabolism in exercising muscle. Inherited diseases reveal the secret of nature ...' (Mineo & Tarui, 1995). In essence, the use of patients with *AMPD1* deficiency to study the role of the PNC in TCA cycle anaplerosis represents a form of human knockout experimentation.

To date, there has only been one investigation comparing skeletal muscle metabolism in healthy controls and AMPD deficient patients during exhaustive exercise (Sinkeler *et al.* 1987). Given the uncertainty of the role for the PNC in muscle metabolism, and the suggestion that dysfunction of the PNC limits exercise capacity in AMPD deficiency (Sabina *et al.* 1980), we directly measured the contribution of the PNC to TCA cycle anaplerosis by examining patients with partial and complete deficiency of AMPD activity. A secondary issue addressed in the current study was the evaluation of the possible causes of 'functional' myoadenylate deficiency mentioned above.

METHODS

The study was reviewed and received approval from the McMaster Medical Center Ethics Review Committee and conformed to the Declaration of Helsinki guidelines. All subjects gave informed written consent to the procedures.

Subjects/patients

Patients who met criteria for complete (homozygous; $n = 3$) and partial (heterozygous; $n = 4$) myoadenylate deaminase (AMPD) deficiency were recruited from the Neuromuscular Clinic at McMaster University Medical Center. The classification was based upon the following criteria: homozygous – absent histochemical stain for AMPD activity plus failure of ammonia to increase on forearm ischaemic testing plus homozygous for the C34T transition mutation in *AMPD1* in whole blood (see below); heterozygous – positive histochemical stain for AMPD activity plus ammonia increase on forearm ischaemic testing that was < 10% of normal control values (with a normal lactate rise) plus heterozygous for the *AMPD1* mutation as above, and no other neuromuscular disorder as ruled out by the screening process outlined below (Fishbein *et al.* 1978, 1990). Forearm ischaemic testing was performed as previously described (Tarnopolsky *et al.* 1998). We also recruited 12 age and sex matched control subjects (control) from the clinic who were investigated for symptoms of muscle fatigue and/or myalgia, and were subsequently found to have a completely normal work-up for neuromuscular disease including: neurological examination, plasma creatine kinase activity, plasma TSH, B_{12}, complete blood count, electrolytes, electromyography and nerve conduction studies, lactate and ammonia response on forearm ischaemic testing, normal light and electron microscopic evaluation of skeletal muscle, normal AMPD activity on a histochemical assay, and did not have an allele with the C34T mutation. Subject characteristics are presented in Table 1.

Design

The subjects arrived at the laboratory following an overnight fast. Upon arrival, subjects were weighed, and a 20 gauge plastic catheter was inserted into the antecubital vein for blood sampling. A 10 ml blood sample was collected into pre-chilled, heparinized tubes and

Table 1. Subject characteristics

Group	Homozygous ($n = 3$)	Heterozygous ($n = 4$)	Control ($n = 12$)	Significance
Age (years)	38 (19)	43 (8)	36 (16)	n.s.
Height (cm)	169 (7)	173 (6)	170 (10)	n.s.
Weight (kg)	69.3 (10.6)	79.4 (13.6)	71.4 (10.4)	n.s.

Values are means (S.D.); not significant (n.s., $P > 0.05$).

centrifuged immediately (200 *g* for 5 min), and plasma lactate concentration was determined using an enzymatic analyser (Yellow Springs Instruments, YSI 23L, Yellow Springs, OH, USA).

Following this, a muscle biopsy of the vastus lateralis was taken using a suction modified Bergstrom needle under sterile conditions. At the same time, a small incision was made in the ipsilateral thigh, ~10 cm proximal to the original incision. This was then closed with sterile gauze and paper tape and subsequently used for the post-exercise biopsy (see below). The muscle sample (125–200 mg) was blotted and quick-frozen in liquid nitrogen.

The subjects then performed a progressive cycling test to voluntary exhaustion on an ergometer (Lode, Netherlands). Exhaustion was defined as the inability to maintain 60 revolutions per minute for 20 s in spite of vigorous verbal encouragement. The initial workload was set at 45 W for 4 min, followed by 15 W increments every 2 min until voluntary exhaustion. During exercise, the subjects had continuous measurement completed for oxygen uptake ($\dot{V}_{O_2}$), carbon dioxide production ($\dot{V}_{CO_2}$), respiratory exchange ratio (RER), and minute ventilation ($\dot{V}_E$) using an open circuit system as previously described (Tarnopolsky *et al.* 1995). Heart rate was also monitored continuously using a telemetry monitor (Polar Electro, Woodbury, NY, USA). When the subjects were close to exhaustion, the paper tape was removed and the leg was re-cleansed while the subject rode. At voluntary exhaustion, the subject was supported on the bike with the leg extended and held by two other assistants while the leg was quickly re-sterilized and a muscle biopsy completed within 4 s of exercise termination. A post-exercise blood sample was taken and analysed as described above. Pressure was held on the incision site and the subject was assisted off the cycle, while the biopsy was processed as described above. The muscle biopsies were stored at −70 °C for < 2 months before being analysed for AMPD activity, nucleotides, amino acids, phosphocreatine (PCr), creatine (Cr), and several TCA cycle intermediates (fumarate, malate and citrate) as outlined below.

Genotype analysis (PCR)

Genomic DNA was isolated from whole blood and the region surrounding exon 2 of the *AMPD1* gene was amplified using a modification of previously described methods (Norman *et al.* 1998). Briefly, the Chelex resin-extracted DNA (Jarnopolsky *et al.* 1998) was amplified using primers (forward-5′-CTTCATACAGCTG-AAGAGACA-3′; reverse-5′-GAATCCAGAAAAGCCATGAGC-3′) designed to create an *Nsp*I restriction site when the C34T transition mutation was present. Amplification was completed using one cycle of 5 min at 95 °C (hot start), followed by 30 cycles of 95 °C denaturing for 20 s, 56 °C annealing for 20 s and 72 °C extension for 45 s, with a final cycle of 72 °C for 7 min (Progene, Techne (Cambridge) Ltd, Duxford, UK). One-tenth of the PCR amplimer mixture was run on a 2% agarose gel (100 V for 30 min) to visualize the 214 bp product. After confirmation that the 214 bp product was present, the amplimers were extracted from the mixture using chloroform–ethanol and resuspended in 10 μl of DNase-free water, and the amplimer mixture was digested with 2.0 U of *Nsp*I overnight at 37 °C in a buffer supplied by the manufacturer (Gibco BRL, Gaithersburg, MD, USA). About 90% of the digested product was then run on a 2% agarose gel (100 V for 30 min). When the C34T transition mutation is present, the *Nsp*I endonuclease cuts the 214 bp amplimer into a 191 and 23 bp product. Thus, the homozygous patients (−/−) had a 191 product only (the smaller product is not consistently visible at the bottom of the gel), the heterozygous patients (+/−) had a 191 and a 214 bp product, while the non-affected controls (+/+) had only the 214 bp product.

Muscle analyses

Muscle AMPD enzymatic activity. Portions (10–20 mg wet weight) of muscle biopsies were homogenized (1:19, w:v) in ice cold 100 mM KCl, 10 mM reduced glutathione and 50 mM Imidazole, pH 7.0. An aliquot of the whole homogenate was retained and the remainder was centrifuged (13 000 *g* for 1 min, at 4 °C) to separate soluble from particulate cell material. The supernatant (soluble cell fraction) was recovered, centrifuged again and retained. AMPD capacity (V_{max}, enzyme activity at near-saturating substrate concentration; 15 mM AMP at pH 7.0 and 30 °C) was determined as previously described in detail (Rush *et al.* 1998). Briefly, a 10 μl aliquot of the whole homogenate or the soluble cell fraction was added to 990 μl of reaction buffer consisting of 150 mM KCl, 15 mM adenosine monophosphate (AMP), and 50 mM Imidazole, pH 7.0, pre-warmed to 30 °C. The reactants were mixed by vortex and the reactions proceeded for 5 min at 30 °C. Reactions were terminated by the addition of 5 volumes of 4.5% (w/v) perchloric acid. Acid extracts were neutralized (KOH/triethanolamine), and the reaction product, inosine monophosphate (IMP), was determined by reversed-phase high performance liquid chromatography (Tullson *et al.* 1990). The particulate cell fraction AMPD capacity was calculated by subtraction of the determined soluble cell fraction AMPD capacity from that of the whole homogenate. Under conditions used for the data reported, this assay system produced IMP linearly with respect to both time and volume of extract used.

Muscle nucleotide/nucleoside and base analysis. Portions (5–10 mg wet weight) of muscle biopsies were homogenized in ice cold 4.5% (w/v) perchloric acid, and the extracts were centrifuged (13 000 *g* for 7 min at 4 °C) to pellet acid-precipitable material. Supernatants were recovered, and neutralized with 0.5 M tri-*n*-octylamine in 1,1,2-trichloro-trifluoroethane. Neutralized extracts were centrifuged (13 000 *g* for 7 min at 4 °C), and the aqueous (upper) phase was recovered for purine analysis. Muscle nucleotides (ATP, ADP, AMP, IMP), nucleosides (adenosine, inosine), and bases (hypoxanthine, xanthine, adenine) were quantified by reversed-phase high performance liquid chromatography, using procedures identical to those previously described (Tullson *et al.* 1990).

TCA intermediates, phosphocreatine and creatine. A 3–8 mg portion of lyophylized muscle was extracted into 0.5 M perchloric acid (containing 1 mM EDTA), neutralized with 2.2 M $KHCO_3$, and assayed for phosphocreatine, creatine, citrate, malate and fumarate using enzymatic methods (Passoneau & Lowry, 1993) adapted for fluorometry (Hitachi F-2000 fluorescence spectrophotometer,

Table 2. Cycle ergometry testing characteristics

	Group			
	Homozygous	Heterozygous	Control	Significance
$\dot{V}_{O_2,peak}$ (l min^{-1})	1.3 (0.5)	1.6 (0.6)	1.7 (0.5)	n.s.
RER	1.09 (0.10)	1.17 (0.12)	1.13 (0.12)	n.s.
Peak power (W)	110 (23)	118 (69)	130 (49)	n.s.
Heart rate (beats min^{-1})	137 (23)	157 (13)	158 (27)	n.s.
Time to fatigue (s)	680 (39)	868 (572)	925 (362)	n.s.
Lactate (mmol l^{-1}): pre	0.69 (0.23)	0.67 (0.28)	0.63 (0.24)	n.s.
post	1.87 (0.60)*	3.20 (1.11)*	3.40 (1.27)*	n.s.

Values are means (S.D.). RER, respiratory exchange ratio. Values of $\dot{V}_{O_2,peak}$, peak power, RER and heart rate were taken at voluntary exhaustion. pre, pre-exercise; post, post-exercise. * Significant increase with exercise ($P < 0.001$). n.s., not significant ($P > 0.05$).

Table 3. Muscle AMPD activity, nucleotides, nucleosides, bases and energy charge

		Group					
		Homozygous		Heterozygous		Control	
AMPD activity	Total	1.43	(0.34)	59.31	(8.26)	154.30	(34.36) [a]
(μmol (g wet wt)$^{-1}$ min^{-1})	Soluble	1.14	(0.24)	39.10	(10.52)	107.47	(28.72) [a]
ATP (μmol (g wet wt)$^{-1}$)	pre	5.2	(0.6)	4.3	(0.7)	5.0	(0.6)
	post	5.2	(1.0)	3.5	(0.8) [b]	3.9	(0.5) [b]
ADP (μmol (g wet wt)$^{-1}$)	pre	0.82	(0.11)	0.77	(0.09)	0.86	(0.07)
	post	0.92	(0.14)	0.76	(0.12)	0.77	(0.11)
AMP (μmol (g wet wt)$^{-1}$)	pre	0.03	(0.02)	0.06	(0.03)	0.05	(0.07)
	post	0.19	(0.20) [c]	0.03	(0.03)	0.05	(0.04)
Energy charge	pre	0.93	(0.00)	0.91	(0.01)	0.92	(0.01)
	post	0.89	(0.06)	0.90	(0.02)	0.91	(0.02)
IMP (μmol (g wet wt)$^{-1}$)	pre	0.01	(0.01)	0.07	(0.03)	0.07	(0.04)
	post	0.01	(0.00)	0.24	(0.14)	0.36	(0.21) [d]
Xanthine (μmol (g wet wt)$^{-1}$)	pre	0.06	(0.01)	0.06	(0.02)	0.06	(0.02)
	post	0.06	(0.01)	0.09	(0.05)	0.09	(0.04)
Adenosine (μmol (g wet wt)$^{-1}$)	pre	0.004	(0.002)	0.005	(0.005)	0.008	(0.007)
	post	0.005	(0.006)	0.003	(0.002)	0.006	(0.006)
Inosine (μmol (g wet wt)$^{-1}$)	pre	0.004	(0.001)	0.008	(0.001)	0.012	(0.011)
	post	0.030	(0.024) [e]	0.113	(0.110) [e]	0.096	(0.06) [e]
Hypoxanthine (μmol (g wet wt)$^{-1}$)	pre	0.023	(0.013)	0.022	(0.013)	0.025	(0.020)
	post	0.043	(0.040) [d]	0.077	(0.079) [d]	0.050	(0.023) [d]
Uric acid (μmol (g wet wt)$^{-1}$)	pre	0.02	(0.00)	0.0	(0.01)	0.02	(0.02)
	post	0.04	(0.01) [e]	0.05	(0.05) [e]	0.04	(0.01) [e]

Values are means (S.D.). pre, pre-exercise; post, post-exercise. [a] Each group's enzyme activity was significantly different from each other ($P < 0.001$); [b] significant decrease with exercise ($P < 0.01$); [c] significant increase with exercise ($P < 0.05$); [d] significant increase with exercise ($P < 0.05$); [e] significant increase with exercise ($P < 0.01$).

Hitachi Instruments). To correct for differences in non-muscle elements between samples, muscle metabolites were corrected to the highest total creatine value obtained within two biopsy samples for a given subject.

Muscle amino acid analysis. A 2–4 mg portion (dry weight) of the biopsy was glass homogenized for 1 min in 100 μl of deionized (Milli Q) water and was then centrifuged for 3 min at 1200 *g*. The supernatant was used for free amino acid analysis by HPLC (Waters Corp., Milford, MA, USA) according to a modified method (Graham *et al.* 1995), first described by Heinrikson & Meredith (1984).

Statistics

Descriptive data between the groups were analysed using a one-way analysis of variance (ANOVA). The muscle data were analysed using two-way ANOVA with a between (groups, 3 levels) and within (pre/post) design. Pair-wise comparisons were made using a Tukey *post hoc* test when significant *F* ratios were obtained. All data in tables and figures are presented as means (±S.D.). A value of $P < 0.05$ was considered to be statistically significant. All data were analysed using a commercially available statistical program (Statistica v. 5.1, StatSoft, Inc., Tulsa, OK, USA).

Table 4. Concentrations of muscle TCA cycle intermediates, phosphocreatine and creatine (mmol (kg dry wt)$^{-1}$)

		Group		
		Homozygous	Heterozygous	Control
Fumarate	pre	0.05 (0.00)	0.08 (0.03)	0.06 (0.02)
	post	0.42 (0.04)*	0.50 (0.13)*	0.46 (0.13)*
Malate	pre	0.49 (0.11)	0.57 (0.18)	0.51 (0.16)
	post	2.90 (0.98)*	2.29 (0.54)*	2.24 (0.42)*
Citrate	pre	0.26 (0.09)	0.31 (0.14)	0.31 (0.16)
	post	0.63 (0.27)*	0.64 (0.25)*	0.65 (0.31)*
Phosphocreatine	pre	85.9 (8.8)	79.3 (9.9)	88.3 (8.7)
	post	61.6 (14.9)†	58.7 (8.8)†	51.8 (10.6)†
Creatine	pre	42.3 (15.2)	45.0 (7.9)	37.5 (5.2)
	post	66.5 (17.5)*	59.3 (13.6)*	63.5 (17.0)*

Values are means (S.D.). pre, pre-exercise; post, post-exercise. * Significant increase with exercise ($P < 0.001$); † significant decrease with exercise ($P < 0.001$).

RESULTS

Cycle ergometry

There were no differences in peak $\dot{V}_{O_2}$, $\dot{V}_E$, $\dot{V}_{CO_2}$, RER, peak power (watts), heart rate, or time to fatigue between the groups (Table 2). Plasma lactate increased similarly for all groups in response to the ergometry testing ($P < 0.001$; Table 2).

Muscle AMPD activity

The enzyme activity for the homozygous group was significantly lower than both the heterozygous and control groups (1.43 *vs.* 59.31 *vs.* 154.30 μmol (g wet wt)$^{-1}$ min^{-1}, respectively, $P < 0.001$). The activity for the homozygous group was 0.9% of that for the control group and the heterozygous group showed 38% of control activity (Table 3). The ratio of soluble cell fraction:total AMPD activity was 0.78 (S.D., 0.05), 0.66 (0.06) and 0.69 (0.05) for the homozygous, heterozygous and control groups, respectively.

Muscle nucleotide/nucleoside and base analysis

Muscle ATP concentration was maintained for the homozygous group before and after exercise, yet showed a significant decrease for the heterozygous and control groups ($P < 0.01$). ADP concentration and cellular energy charge ($[ATP] + \frac{1}{2}[ADP]/[ATP] + [ADP] + [AMP]$) were unchanged before and after exercise and were not different between groups. There was a trend towards a drop in energy charge with exercise for all groups ($P = 0.052$). AMP concentration was not different before or after exercise for the heterozygous and control groups, yet it increased significantly due to exercise for the homozygous group ($P < 0.05$). IMP concentration was unchanged with exercise for the homozygous and heterozygous group, yet increased significantly for the control group ($P < 0.05$). There was a trend for IMP to increase for the heterozygous group ($P = 0.072$). Xanthine and adenosine concentrations did not change with exercise for any of the groups, whereas inosine ($P < 0.01$), hypoxanthine ($P < 0.05$) and uric acid concentrations ($P < 0.01$) increased similarly for all groups (Table 3).

TCA intermediates, phosphocreatine and creatine

The concentration of each of the three TCA cycle intermediates (Table 4) and the sum of the three TCAi intermediates (**Σ**TCAi; Fig. 1) increased similarly for each of the groups following exercise ($P < 0.001$). Resting phosphocreatine (PCr) and creatine (Cr) concentrations were similar between groups. PCr decreased significantly and similarly for all groups ($P < 0.001$), with a reciprocal increase in Cr ($P < 0.001$) (Table 4).

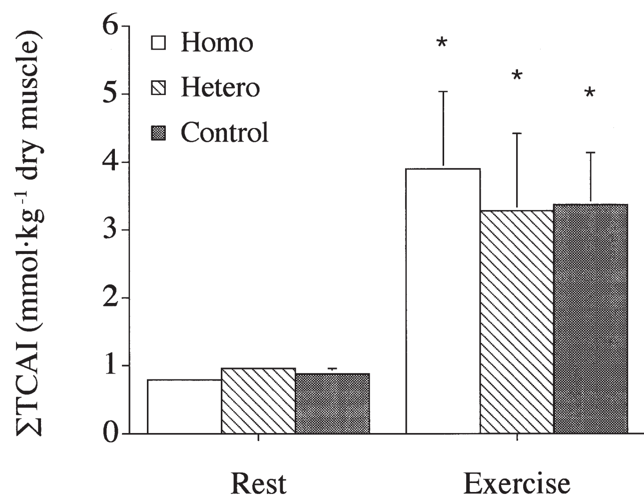

Figure 1. Sum of concentration of intramuscular TCA cycle intermediates at rest and after exercise

Values represent the sum of citrate + malate + fumarate concentrations, expressed as means ± S.D. * Significantly different ($P < 0.001$) *vs.* within same age group.

Table 5. Selected muscle amino acid concentrations (mmol (kg dry wt)$^{-1}$)

		Group		
		Homozygous	Heterozygous	Control
Alanine	pre	13.7 (5.4)	12.9 (2.4)	11.8 (3.2)
	post	16.7 (2.4)	14.6 (5.2)	13.8 (2.8)
Aspartate	pre	0.67 (0.32)	0.78 (0.47)	0.98 (0.35)
	post	0.79 (0.21)	0.84 (0.23)	0.94 (0.46)
Taurine	pre	81.8 (10.1)*	54.0 (9.9)	42.9 (10.8)
	post	59.1 (14.4)*	51.5 (15.3)	43.1 (10.9)
Glutamine	pre	81.8 (10.1)*	49.7 (13.7)	53.5 (11.2)
	post	63.9 (5.7)*	43.6 (9.4)	51.7 (9.8)
Glutamate	pre	16.4 (5.1)	12.1 (2.1)	12.5 (3.2)
	post	8.1 (3.8)†	9.9 (2.7)†	8.4 (3.7)†

Values are means (S.D.). pre, pre-exercise; post, post-exercise. * Significantly higher for homozygous ($P < 0.05$); † significantly lower after exercise ($P < 0.05$).

Muscle amino acids

There were no between-group differences in any of the nine essential amino acid concentrations (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), nor was there a significant effect of exercise upon these amino acids (data not shown). For the non-essential amino acids there were no between-group nor exercise effects for alanine, asparagine, serine, ornithine, tyrosine, proline, aspartate, glycine and arginine. Muscle glutamate decreased significantly and similarly for all groups as a result of the exercise (Table 5). There was a significantly higher taurine concentration for the homozygous compared with heterozygous and control groups (main effect for group, $P < 0.05$; Table 5). A similar difference was found for glutamine concentration with the homozygous group showing higher overall concentrations as compared with heterozygous and control groups (main effect for group, $P < 0.05$, Table 5).

DISCUSSION

A major finding in the current study was the ability of the muscle to maintain TCA cycle intermediate (TCAi) concentrations and cellular energy charge during exercise in spite of AMPD activity being less than 1 % of control values. This finding demonstrates the utility of using an inborn error of metabolism to unravel a fundamental question in muscle physiology, namely, that the PNC is not required for TCA cycle anaplerosis. From a clinical perspective, the main finding in this study is that patients with less than 1 % residual AMPD activity were not markedly different from subjects with normal AMPD activity in their tolerance for progressive cycle ergometry exercise.

One conceivable consequence of AMPD deficiency would be an impairment of the PNC with a resultant attenuation of TCAi anaplerosis (Sabina *et al.* 1980; Flanagan *et al.* 1986). It has previously been demonstrated that the total muscle TCAi content increased severalfold during the initial minutes of moderate to intense contraction, but the TCAi gradually declined when the bout was prolonged (Sahlin & Broberg, 1990; Gibala *et al.* 1997*b*). The PNC results in the re-amination of IMP to AMP and is catalysed by adenylosuccinate synthetase and adenylosuccinate lyase, respectively: IMP + aspartate + GTP → adenylosuccinate + GDP + P_i → AMP + fumarate. Thus, fumarate production and TCAi anaplerosis could depend upon AMPD and PNC activity. Using 5-amino-4-imidazolecarboxamide ribose to inhibit the adenylosuccinate lyase reaction, investigators have reported that energy production did not meet energy demand in exercising rodents (Flanagan *et al.* 1986). This group also reported that lactate production was unimpaired and concluded that inhibition of the PNC must have caused aerobic energy impairment (Flanagan *et al.* 1986). Another group used the adenylosuccinate synthetase blocker, hadacidin, to study *in situ* rodent muscle exposed to various frequencies of tetanic stimulation and found no impairment of contractile force, nor any effects upon PCr hydrolysis, or lactate production (Meyer & Terjung, 1980). A case report in a patient with biochemically confirmed AMPD deficiency found a severe decrease in ATP and an increase in adenosine concentration following exercise, and a very prolonged ATP resynthesis rate (~50 % normal by 3 h of recovery) (Sabina *et al.* 1980). This group hypothesized that this observation was due to a 'Disruption of the purine nucleotide cycle' (Sabina *et al.* 1980). It is difficult to ascribe this profound drop in ATP content and such an impairment of ADP rephosphorylation to AMPD deficiency, as neither the current study nor another study of ATP/ADP content (Sinkeler *et al.* 1987) found significant ATP depletion following exercise. It could be that the patient in this single case report (Sabina *et al.* 1980) had other confounding factors that led to this profound disruption in cellular energy status and would be considered to have coincidental AMPD deficiency.

The results of the current study have demonstrated that near total AMPD enzyme deficiency did not result in an

impairment of exercise-induced anaplerosis of the TCAi pool. The magnitude of the increase in the TCAi pool and the strength of the statistical analysis very strongly support that this is a real finding and not a false positive result. This finding is consistent with the concept that the PNC is not quantitatively important in exercise-mediated TCAi anaplerosis, and supports the previously demonstrated importance of the alanine aminotransferase (ALT) reaction for TCAi anaplerosis (Sahlin *et al.* 1990; Gibala *et al.* 1997*b*). It could be argued that changes in three of the eight TCA cycle intermediates may not be representative of the total TCAi pool. However, we (Gibala *et al.* 1997*b*) have found that the sum of citrate, malate and fumarate accounts for approximately 70% of total pool size, and they increase in a direction similar to that of other TCA cycle intermediates at the start of exercise (except for 2-oxoglutarate, which decreases or does not change markedly). From a fatigue perspective, a depletion of TCAi cannot account for contractile failure with this type of exercise, for the TCAi pool remained above resting levels following exercise in all groups at exhaustion in the current study.

The amino acid data from the current study demonstrated a decrease in glutamate concentration with exercise that was similar to that reported by Gibala *et al.* (1997*b*). Although we did not find a statistically significant increase in alanine concentration in the current study (up 20%), it was directionally similar to that reported in recreationally exercise-trained males (up 54%) (Gibala *et al.* 1997*b*). The amino acid data support the hypothesis that the ALT reaction is important for TCAi expansion during exercise in healthy young controls (Gibala *et al.* 1997*b*), and in patients with partial and complete muscle AMPD enzyme deficiency. The increase in the content of glutamine and taurine in the patients with complete AMPD deficiency is difficult to explain. Given that glutamine and taurine did not change before or after exercise, it is not likely that these changes are of any significance to energy transduction under high intensity exercise conditions.

In theory, an increase in phosphocreatine (PCr) hydrolysis may increase in response to an impairment of one of the energy generating pathways. Such is the case with mitochondrial cytopathies, where impaired aerobic energy transduction is accompanied by increased glycolytic/glycogenolytic flux (increased lactate) and an increase in PCr hydrolysis (Tarnopolsky, *et al.* 1998; Tarnopolsky & Parise, 1999). Furthermore, an enhanced PCr hydrolysis has been described in patients with McArdle's disease during exercise (Sahlin *et al.* 1995). Together, these results suggest that high grade aerobic and anaerobic metabolic defects are partially compensated for by an enhanced PCr hydrolysis. In the current study, there were no between-group differences in PCr hydrolysis in response to exercise, nor were the basal concentrations reduced in the AMPD deficient patients. Although it is possible the shorter exercise intensity on the ergometer for homozygous patients could have masked a greater PCr hydrolysis for this group, our findings are consistent with an earlier study comparing five AMPD deficient patients to ten control subjects during ischaemic, isometric exercise (Sinkeler *et al.* 1987). The lack of an enhanced PCr hydrolysis (in combination with normal lactate production) during cycling exercise in AMPD deficiency suggests that AMPD deficiency does not result in an upregulation of compensatory anaerobic energy transduction pathways during this kind of exercise.

A recent paper has demonstrated a reduction in the force of contraction of mechanically skinned rat skeletal muscle with 0.4 and 3 mM adenosine (Blazev & Lamb, 1999). This group has suggested that an increase in adenosine concentration could account for the fatigue experienced by patients with AMPD deficiency (Blazev & Lamb, 1999). This group (Blazev & Lamb, 1999) quotes a case report where one patient showed a post-exercise increase in adenosine (Sabina *et al.* 1980) as evidence for the potential role in fatigue. As we have argued above, the single case report does not have muscle biochemical findings that are consistent with other larger case series (Sinkeler *et al.* 1987), nor with the results of the current study. Given that we did not observe a significant increase in adenosine in the AMPD deficient subjects from the current study, we do not feel that adenosine causes muscle fatigue during this type of exercise.

The biochemical data reported in the current study in the patients with high grade AMPD deficiency support the conclusions that AMPD deficiency is a 'harmless genetic variant' (Verzijl *et al.* 1998). This group (Verzijl *et al.* 1998), and others (Norman *et al.* 1995, 1998), reported that complete AMPD genetic deficiency occurs in about 2% of an otherwise asymptomatic group of subjects. Furthermore, the allelic frequency (heterozygous state) is between 13.7 and 18% in asymptomatic healthy controls in the Netherlands (Verzijl *et al.* 1998) and the United States/Sweden (Norman *et al.* 1995, 1998). From a phylogenetic perspective, it would be difficult to believe that a genetic mutation that led to significant muscle dysfunction would continue to have such a high allelic frequency in out-bred populations.

Some have suggested that AMPD deficiency is found with a higher frequency in patients referred for exercise-induced myalgias (Kelemen *et al.* 1982; Gross, 1998). This observation was called into question based upon the results of Verzijl *et al.* (1998) who found similar homozygous frequency rates for patients with exertional myalgia (1%, $n = 1836$ patients) and neuromuscular complaints (1.8%, $n = 2388$ patients). One possible explanation for the discrepancy in the results could be the fact that the diagnosis of AMPD deficiency was based upon forearm ischaemic testing and histochemical AMPD activity

where 8.3 % of the patients with exertional myalgia were completely deficient (Kelemen *et al.* 1982). We have found that the combination of the forearm ischaemic test and the AMPD histochemical staining technique may result in a false positive diagnosis of 'primary' AMPD deficiency (Tarnopolsky *et al.* 1999). For example, in the current study we had identified six patients who could be classified as having primary AMPD deficiency using solely forearm ischaemic testing and muscle histochemistry for AMPD activity, yet only three had absent biochemical enzyme activity, one had a partial reduction in activity (~50 %) and two had normal enzyme activity. The genetic analysis was 100 % concordant with the direct biochemical assay used in the current study. Therefore, we feel that either direct biochemical enzyme activity determination and/or genetic analysis are required to classify patients as having 'primary' AMPD deficiency. The use of correlative associations between AMPD deficiency and symptoms has led to an ongoing debate about the pathogenicity of the enzyme deficiency (Fishbein, 1999). For this reason, we chose to examine the physiological and muscle metabolic responses to high intensity exercise after rigorously assessing the AMPD enzyme activity of each subject through direct biochemical assay, to objectively determine whether AMPD enzymatic deficiency had a negative impact upon muscle metabolism/fatigue. We do concede that with the variation in exercise duration, and hence intensity, and the small number of subjects, we may not have detected a negative effect of AMPD deficiency on performance (type II error). It is probable that if more than three homozygous subjects had been tested then a statistically significant difference in performance may have been revealed, albeit unrelated to impaired TCAi depletion. However, the biochemical data from the current study and from a previous study using isometric exercise (Sinkeler *et al.* 1987) provide strong support against a role for the pathways thought to be altered by AMPD deficiency that could alter exercise performance. It is possible, however, that the similar decrease in PCr hydrolysis and somewhat shorter exercise duration for the homozygous subjects, as compared with control subjects, could indicate a greater PCr hydrolysis per unit of time in the presence of AMPD deficiency. Future studies should evaluate exercise performance in a large number of patients with genetically or biochemically proven AMPD mutations/deficiency to rule out a more subtle effect of the defect. If one is found, it must be through a pathway unrelated to TCAi anaplerosis. A final finding in the current study is that the patients who met Fishbein's criteria for functional AMPD deficiency (Fishbein *et al.* 1990) did not appear to have a K_m mutant but instead had enzyme activities (< 50 %) consistent with their heterozygous state for the AMPD allele. If the heterozygous subjects had a K_m mutant that was not activated *in vivo*, we would have expected to see an attenuation of IMP accumulation and/or an increase in AMP accumulation following exercise. These findings do not rule out that some patients who meet the criteria for functional AMPD deficiency may have a K_m mutant enzyme or an impairment of ammonia efflux (Fishbein *et al.* 1990). However, the high frequency of the mutant allele would suggest that the most likely scenario would be heterozygosity for the *AMPD1* mutant allele. We also considered that an abnormality of the soluble:total AMPD enzyme activity ratio could possibly explain some cases of functional AMPD deficiency, for one study found three regions in the AMPD1 peptide that regulated protein binding (Hisatome *et al.* 1998). However, our data have shown that the ratio of soluble:total AMPD activity was similar in all groups (~0.70). This indicated that protein binding is not affected by the *AMPD1* mutation, yet does not rule out the remote possibility that some cases of functional AMPD deficiency are due to a mutation in one of the three regions that are important in protein binding (Hisatome *et al.* 1998).

In summary, a high grade deficiency of AMPD activity does not result in an apparent impairment of TCAi anaplerosis, exercise capacity, phosphocreatine hydrolysis, or cellular energy charge during exhaustive cycling exercise.

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