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Differential effects of creatine depletion on the regulation of enzyme activities and on creatine-stimulated mitochondrial respiration in skeletal muscle, heart, and brain

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Abstract

Guanidinopropionic acid (GPA), an analogue of creatine (Cr), is known to inhibit Cr uptake by cells. The metabolic effects of chronic Cr depletion on brain, heart and soleus muscle of rats were studied. In GPA hearts and soleus muscle, total specific creatine kinase (CK) activity was decreased by approx. 40% compared to controls, whereas in brain this same activity was elevated by a factor of two. Immunoblot analysis of soleus mitochondria from GPA rats showed an approximate 4-fold increase in Mi-CK protein and a concomitant 3-fold increase in adenine nucleotide translocator (ANT) protein, when compared to control. In GPA-fed rats, the specific activities of adenylate kinase (ADK) and succinate dehydrogenase were significantly higher in brain and soleus (2-fold), but heart remained the same. However, hexokinase (HK) decreased by approx. 50% both in heart and soleus, indicating that muscle and brain follow different strategies to compensate the energy deficit caused by creatine depletion. Skinned muscle fibres from Cr-depleted soleus attained approx. only 70% maximum state 3 respiration with 0.1 M ADP in the presence of 10 mM Cr compared to 100% in control fibres. This defect in Cr stimulated respiration was also seen in isolated heart mitochondria, but was normal in those from brain. The observed deficit of Cr-stimulated respiration, the significant accumulation of Mib-CK and ANT, concomitant with the formation of Mib-CK rich intra-mitochondrial inclusions shown by electron microscopy, indicate that Mib-CK function and coupling to oxidative phosphorylation (OXPHOS), is impaired in these abnormal mitochondria. In addition, our results show tissue-specific metabolic compensations to Cr depletion.

Keywords: Guanidino propionic acid; Creatine; Mitochondrial inclusion body; Creatine kinase; Adenine nucleotide translocator; Metabolism; Oxidative phosphorylation; (Rat)

1. Introduction

Energy metabolism of brain and muscle during excitation is characterised by an up to 10-fold increase of ATP turnover within milliseconds. It is hypothesised that during these rapid changes, creatine/phosphocreatine (Cr/PCr) is necessary as an energy buffering and transferring system to avoid large fluctuations of cellular ATP/ADP levels [1–4]. The creatine kinase (CK) group of isoenzymes consists of cytosolic dimers, BB-CK (brain-specific), MM-CK (muscle-specific) and a heterodimer of both of these subunits, MB-CK, which is only present in cardiac muscle cytosol. In addition, there are two mitochondrial-specific CKs (Mi-CK) positioned in the intermembrane space, termed Mib-CK (specific for sarcomeric muscle) and Mia-CK (the ubiquitous isoform) [4]. Mitochondrial CK isozymes form cube-like octamers, with a four-fold symmetric structure, two identical top and bottom faces, and a channel through the centre of the molecule [5]. Furthermore, several observations suggest a structural importance for the octameric form, in that the octamer has the ability to link two artificial membranes [6–9] and interact with porin [10].

It has already been shown that patients suffering from mitochondrial myopathies [11] show enlarged mitochondria with paracrystalline inclusion bodies, highly enriched with Mib-CK [12]. These mitochondria are found predomi-

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nantly in the so-called 'ragged red fibres' regularly seen in muscle biopsies from these patients as well as in AIDS patients administered Zidovudine [13]. It is not clear whether these are actually functional or nonfunctional aggregates of Mib-CK.

Similar Mib-CK-rich mitochondrial inclusions are observed in slow twitch muscles of rats exposed to ischemia [14] or depleted of Cr [15,16]. This indicates that chronic lowering of cellular phosphorylation potential is a crucial factor involved in the manifestation of these inclusion bodies. 3–4 months feeding of beta-guanidinopropionic acid (GPA), an analogue of creatine which competitively inhibits creatine uptake [17], leads to creatine depletion. After 3 months feeding of rats with GPA, [PCr], [Cr] and [ATP] are lowered by 90%, 55% and 50% respectively, compared to control animals [18,19]. While GPA is taken up by muscle cells and is phosphorylated via CK to GPAP, it becomes a very poor substrate for CK, having a two-fold higher K_m and 1/1000 V_{max}, leading to a significant decrease in the PCr/ATP phosphagen flux [19]. Consequential biochemical events to Cr depletion have been well documented, the most important being an increase in aerobic capacity [19], an increase in the glucose transporter 4 (GLUT 4) expression [20], a decrease in AMP deaminase activity [21], an increase in glycogen content, a decrease in glycolysis and an increase in slow twitch fibres in skeletal muscle with a subsequent shift in the expression of fast to slow myosin isoforms [19,20]. All these facts emphasize the inherent plasticity of muscle to adapt to imposed metabolic alterations [22].

The aim of this particular study was to investigate furthermore tissue-specific compensatory mechanisms to creatine depletion in skeletal and cardiac muscle, as well as in brain, with emphasis on Mib-CK amount and functionality in the mitochondrial inclusions formed preferentially in red muscle, e.g., soleus.

2. Materials and methods

2.1. Electron microscopy

Muscle samples were cut into 1-mm^3 blocks and primarily fixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4°C for 90 min, washed with cacodylate buffer with 0.15 M sucrose and post-fixed with 2% osmium tetroxide in cacodylate buffer containing 0.11 M sucrose. Fixed samples were then dehydrated via a graded series of ethanol concentrations, for infiltration with and embedding in epon/araldite resin. Heat polymerization occurred at 55°C overnight and ultra-thin sections were cut with a Reichert/Jung Ultracut E. The sections were observed on carbon-coated grids after staining with uranyl acetate (aqueous), and lead citrate, using a Jeol 100C Transmission Electron Microscope. Micrographs were taken at 80 kV.

2.2. GPA production and animal care and feeding

GPA was synthesized in bulk according to the method described by Rowley et al. [23]. Female Sprague-Dawley rats were fed at three weeks of age on control diet or a diet containing 2.5% GPA, and also 1% GPA in their water supply for 3–4 months. Rats were killed using CO_2 or cervical dislocation and then exsanguinated.

2.3. Enzyme assays

Adenylate kinase (EC 2.7.4.3), hexokinase (EC 2.7.1.1), and lactate dehydrogenase (EC 1.1.1.27) were measured in agreement with Bücher et al. [24]. Creatine kinase (EC 2.7.3.2) was determined with creatine and ATP in a coupled enzyme assay using pyruvate kinase/lactate dehydrogenase as the indicator reaction to measure the ADP production. To determine maximal activity in muscle extracts or gradient fractions thereof, the assay system used was hypotonic and contained 50 mM triethanolamine (pH 7.6), 5 mM EDTA, 8 mM $MgCl_2$, 16 mM KCl. To analyse the enzyme under isotonic conditions the mitochondria were suspended in 0.3 M sucrose, 10 mM Hepes (pH 7.4), 8 mM $MgCl_2$, 5 mM succinate. Both assay systems contained 0.2 mg NADH/ml, 1 mM phosphoenol-pyruvate, 2.5 mM ATP, 60 μM rotenone, 30 μg/ml oligomycine, 0.2 IU pyruvate kinase, 0.5 IU lactate dehydrogenase (free of $(NH_4)_2SO_4$), the reaction was started with 20 mM creatine and the oxidation of NADH was followed in a double beam spectrophotometer at 340 nm.

2.4. Preparation of mitochondria from rat brain

Mitochondria from rat brain were prepared by differential centrifugation according to Rehncrona et al. [25] in a medium containing 0.25 M mannitol, 0.075 M sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.4) and 0.1% fatty acid free bovine serum albumin. The mitochondria were further purified by 35 min centrifugation at 38 000 rpm in a 60 Ti rotor (Beckman) on a 20% Percoll gradient. Percoll was removed from the mitochondrial fraction by washing twice with the above medium.

2.5. Preparation of heart muscle mitochondria

Hearts were removed and finely minced with surgical scissors [2] in a buffer solution containing 0.3 M sucrose, 10 mM Na-Hepes and 0.2 mM EDTA, pH 7.2 at 4°C. The muscle mince was then incubated with 125 μg/ml trypsin (Difco) solution and then gently homogenised. This homogenate was then centrifuged at $1000 \times g$ for 10 min and the pellet discarded. The supernatant was centrifuged at $8000 \times g$ for 15 min, and the resultant pellet was resuspended in isolation medium containing 1.0 mg/ml BSA and centrifuged again. The mitochondria were further purified by 35 min centrifugation at 38 000 rpm in a 60 Ti

rotor (Beckman) on a 20% Percoll gradient. Percoll was removed from the mitochondrial fraction, as before, by washing twice in the isolation medium.

2.6. Preparation of soleus mitochondria

Solei were removed from rats and placed into a medium containing 220 mM D-mannitol, 70 mM sucrose, 10 mM Hepes 5 mM BME and 0.2 mM EDTA (MSH buffer), and minced with a surgical scissors. The mince was washed with excess buffer to remove a maximum amount of blood from the tissue. The minced tissue was then ground manually with a glass tissue grinder, and then homogenised at 4°C. The homogenate was centrifuged at $1000 \times g$ for 10 min, and the supernatant removed. The pellet was resuspended in 20 ml MSH buffer and centrifuged again for 10 min. Both supernatants were pooled and centrifuged at $8000 \times g$ for 20 min, the supernatant discarded and the pellet resuspended in fresh MSH buffer. This was again centrifuged at $8000 \times g$ for 20 min and the pellet suspended in 200 μl of buffer and kept for biochemical analysis at 4°C or frozen with liquid nitrogen.

2.7. Gel electrophoresis and Western blots

Protein was determined using the BioRad method and 10% polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of sodium dodecyl sulphate (SDS) according to Laemmli (1970) [26], with 20 μg of mitochondrial protein being loaded onto each lane. After semi-dry blotting, protein transfer efficiency was observed by Ponceau S staining of the nitrocellulose membrane and then the membranes were blocked by a buffer containing 1% BSA in phosphate-buffered saline (PBS: 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2PO_4, 1.4 mM KH_2PO_4, pH 7.4). Antibody labelling was carried out for Mib-CK and ANT [27] at concentrations of 1:1000 and 1:400 respectively. After 1 h the membranes were washed 3 times with the above blocking buffer. The secondary antibody, peroxidase-conjugated goat anti-rabbit IgG, was then applied at 1:3000 concentration, and detected using 4-chloro-1-naphthol and H_2O_2 as substrate, or luminol (2.5 mM luminol, 0.5 mM *p*-iodophenol, 50 mM Tris-HCl pH 7.5, and 0.15% H_2O_2) [28] exposure to X-ray film (Fugi Medical RX) for densitometric analyses, using the Image Quant 3.3 (Molecular Dynamics California).

2.8. Fractional extraction

Musculus soleus was minced in a micro-grinder (Braun Melsungen, Germany). 100 mg of the minced soleus were suspended in a ten-fold volume of a medium containing 0.3 M sucrose, 2 mM EDTA, 20 mM triethanole-amine (pH 7.4). The suspension was stirred for 15 min at 0°C and subsequently centrifuged for 30 min at $300\,000 \times g$ in a Beckman rotor 50 Ti. The supernate (C1) was removed and subjected to enzyme determinations, while the sediment was resuspended and centrifuged after 15 min stirring as before. After withdrawal of the resulting supernate (C2) the sediment was sonicated and stirred for 15 min in a ten-fold volume of 0.1 M Na/K-phosphate buffer (pH 7.4). Subsequently, the supernate (M1) was obtained after centrifugation as described above, the sediment was resuspended by Teflon homogeniser in a 3-fold volume of phosphate buffer to yield fraction (M2).

2.9. Isoenzyme electrophoresis

The isozymes were separated on cellulose acetate strips according to Ishida et al. [29] using a buffer of 50 mM sodium barbital and 2.7 mM EDTA (pH 8.8). The cellulose acetate strips were soaked in the buffer for 20 min and were then blotted free of excess buffer with filter paper. The samples were applied in the middle of the strips and were run for 45 min at 3 mA in a Boskamp chamber. After electrophoresis, the isozymes were visualised by incubation of the strips on top of a 1% agarose gel containing 0.6 M glycyl-glycine (pH 7.0), 0.04 M Mg-acetate, 12 mM creatine phosphate, 3 mM ADP, 1 mM NADP, 10 mM AMP, 0.5 mM nitroblue tetrazolium, 0.2 mM phenazine methosulfate, 20 mM glucose, 5 IU hexokinase and 3 IU glucose 6-phosphate dehydrogenase.

2.10. Preparation of skinned soleus fibres

Solei, from both control and GPA fed animals, were treated in the same way. Soleus muscle was removed after killing the rat and exsanguination, and then placed into the isolation buffer (EGTA 10 mM, $MgCl_2$ 9.5 mM, $KHPO_4$ 3 mM, Taurine 20 mM, K-Mes 49 mM, imidazole 20 mM), pH 7.1, Ca^{2+} 2.27 mM and at 4°C. They were then teased apart to produce fibre bundles of equal size, i.e., 50 mm by 3 mm. Fibres were then introduced to the isolation buffer containing 50 μg/ml digitonin and mixed gently for 20 min at 4°C. Fibres were then removed from this medium and washed with the respiration buffer and maintained at 4°C on ice [30].

2.11. Respiration analyses

The respiration analyses were carried out at 25°C, with a Cyclobios-oxygraph (Anton Paar, Innsbruck, Austria) in the respiration buffer (mannitol 75 mM, Sucrose 25 mM, KCl 100 mM, KH_2PO_4 10 mM, EDTA 0.5 mM, BSA 1 mg/ml, Tris-HCl 20 mM), pH 7.4 [30] and using fibre bundles with a wet weight of 15 mg.

Stimulation of Oxidative Phosphoylation (OXPHOS) was carried out in the presence of 5 mM succinate and 5 mM $MgCl_2$, with 0.1 mM or 2.0 mM ADP, with and without 10 mM Cr present. Maximum State 3 respiration was that which was measured after stimulation with 2.0 mM ADP and all other values were based as percentage of this stimulation.

3. Results

3.1. Electronmicroscopy

Ultrastructural analysis of soleus from GPA fed rats showed the typical sub-sarcolemmal accumulation of abnormal mitochondria, containing parallel sheets of intermembranous inclusion bodies already shown to contain Mi-CK [39] (Fig. 1). The small arrow indicates a regular structure enveloped by cristae membrane, which, based on the similarity with those seen in patients with mitochondrial myopathies [12], could represent multilayers of Mi-CK. Whatever the true structure of these inclusions may be, it is hard to imagine an advantage for normal function. The majority of these inclusion bodies occur between cristae membranes, rather than between the inner and outer membrane. Very few of these particular mitochondria were seen between the myofibrils. It is also evident from the micrograph that numerous glycogen particles are in the surrounding cytoplasm of these altered mitochondria. No such abnormalities were seen in control muscle (figure not shown).

3.2. Mib-CK and ANT Western and immunoblots

Western blots, followed by immuno-staining and densitometry, carried out on isolated mitochondria from soleus muscle of GPA and control rats, for both ANT and Mib-CK, revealed increases in both proteins, (Fig. 2A,B). For ANT, densitometric analysis of immunoblots show a 3-fold increase, in GPA mitochondria (control = 1303 ± 663, and GPA = 3046 ± 1404 ($n = 4$ and 6, respectively)), and 4-

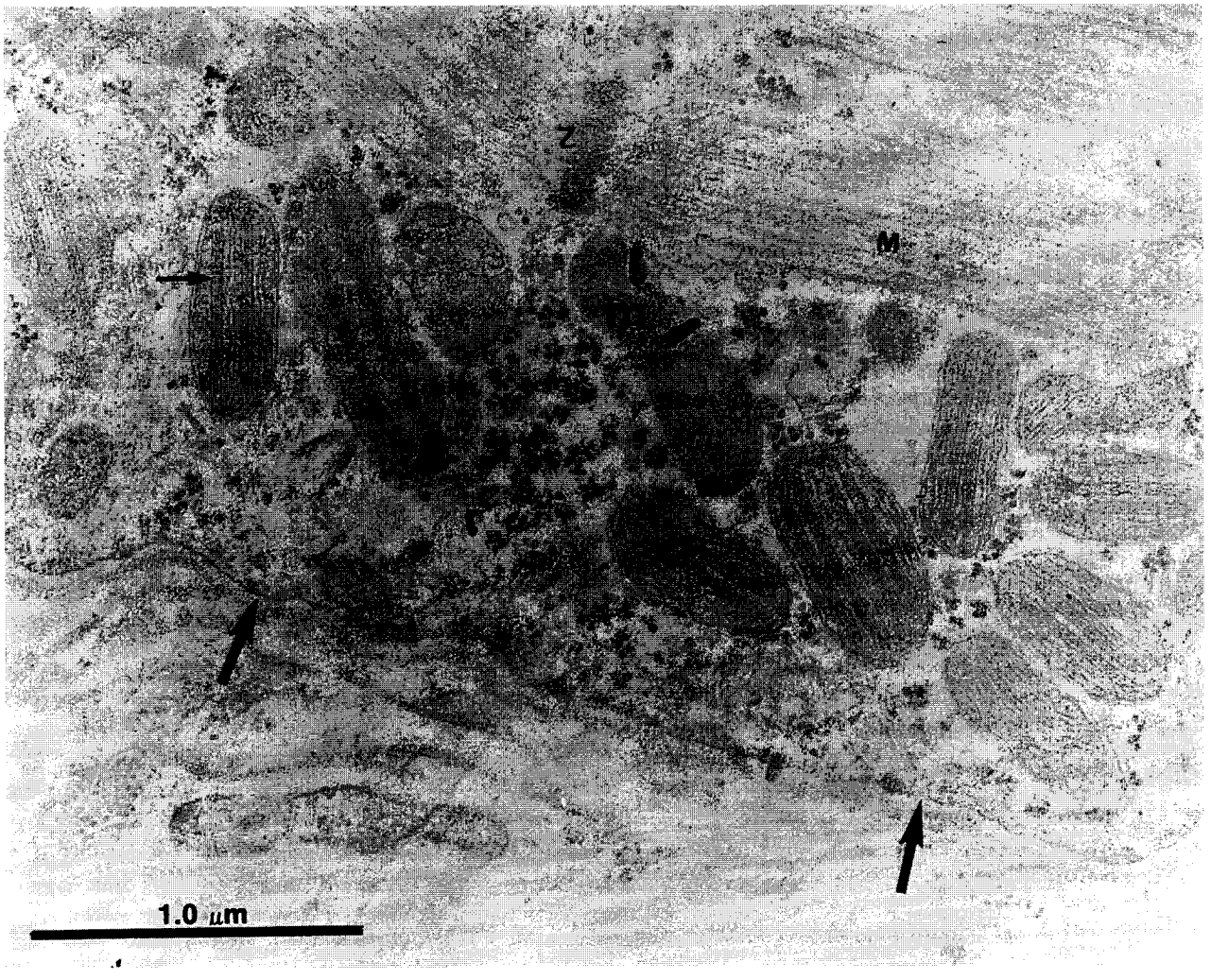

Fig. 1. Electron micrograph of GPA-treated soleus muscle. Soleus fibres were removed from the hind limb of rats fed GPA for 3 months, fixed with 2.5% glutaraldehyde, postfixed with 2% osmium tetroxide, dehydrated through a graded series of alcohol and acetone, and thereafter processed through to Epon resin. Silver sections were then placed onto carbon-coated grids and stained with 2.5% uranyl acetate and 1% lead citrate for enhancement of contrast. Low magnification electron micrograph (48 000) of abnormal mitochondria (marked m) with inclusion bodies (small arrow) at the subsacrolemmal (large arrows) region of a soleus fibre. Myofibril displaying classical M and Z-band architecture, (marked M and Z, respectively). Note the large electron dense deposits of glycogen granules in the cytoplasm around the mitochondria.

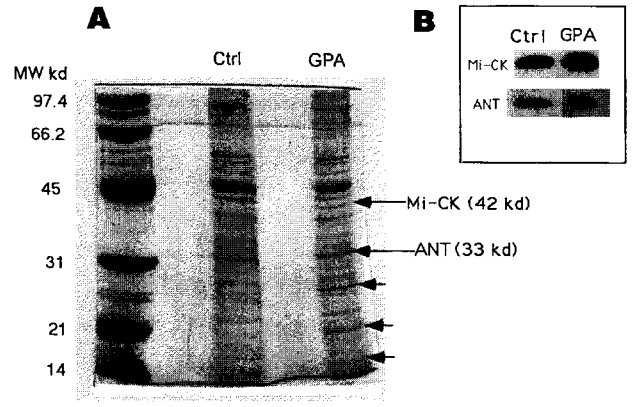

Fig. 2. SDS-PAGE, Coomassie blue staining, and Western blot antibody staining of isolated soleus GPA and control mitochondria. (A) Coomassie blue-stained protein on a 10% SDS-PAGE gel, (with molecular weight marker lane on the left), of 20 μg of mitochondria isolated from GPA-fed rat (middle lane) and control rat (right lane) soleus showing significantly stronger bands in the GPA-lane, at 42 kDa and 33 kDa, corresponding to the apparent MWs of Mib-CK and ANT (large arrows), respectively. Some additional stronger bands corresponding to as yet non-identified proteins of apparent MW of 15, 21 and 25 kDa (small arrows). (B) Box inset contains representative Mib-CK and ANT bands after luminol detection of HRPO-conjugated secondary antibody against the primary anti-Mib-CK and anti-ANT antibodies respectively, which show a clear increase of Mib-CK and ANT in the GPA lanes compared to control. Densitometry on the antibody-stained bands was carried out on the exposed X-Ray film reveals an approximate 3–4-fold and 4–5-fold increase in ANT and Mib-CK, respectively, in the GPA soleus mitochondria over control mitochondria (see Section 3).

fold for Mib-CK (control = 1544 ± 196 and GPA = 7543 ± 1432 (n = 3 for both)) (Fig. 2B). The increase in Mib-CK gives further support to the hypothesis that the inclusion bodies, seen in Fig. 1, consist mainly of this particular enzyme [12,39]. The observed increase in ANT protein may be a compensation to a possible functional interference by the accumulation of Mi-CK in these mitochondria.

3.3. Relative creatine kinase isoenzyme ratios in soleus muscle, heart and brain after chronic GPA treatment

We separated cytosolic from mitochondrial creatine kinases by graded extraction of minced soleus muscle (Fig. 3 left). Stirring the minced muscle twice for 15 min in sucrose/EDTA medium with intermittent centrifugation led to the extracts C1 and C2. As analysed by isozyme electrophoresis (Fig. 3 right), these extracts contained mainly the cytosolic isozyme MM-CK. The mitochondrial isozyme was extracted, in fraction M1, from the muscle sediments after sonification in 0.1 M phosphate buffer followed by 15 min stirring. Isoenzyme electrophoresis showed that fraction M1 contained mostly Mi-CK in addition to some MM-CK. The solei from GPA-treated and control rats were extracted by the same weight to volume ratio. Thus, the experiment in Fig. 3 clearly demonstrates in soleus from GPA-treated rats, a decrease of cytosolic creatine kinase per gram fresh weight and a concomitant increase of the mitochondrial isoenzyme. Whereas total specific activity in GPA soleus is lower by 50%, there is a clear increase in Mib-CK activity (Table 1 and Fig. 3). Heart, like soleus, responded to Cr depletion by a lowering of total specific CK activity but unlike soleus, maintained normal or slightly lower Mib-CK-specific activity (Table 2).

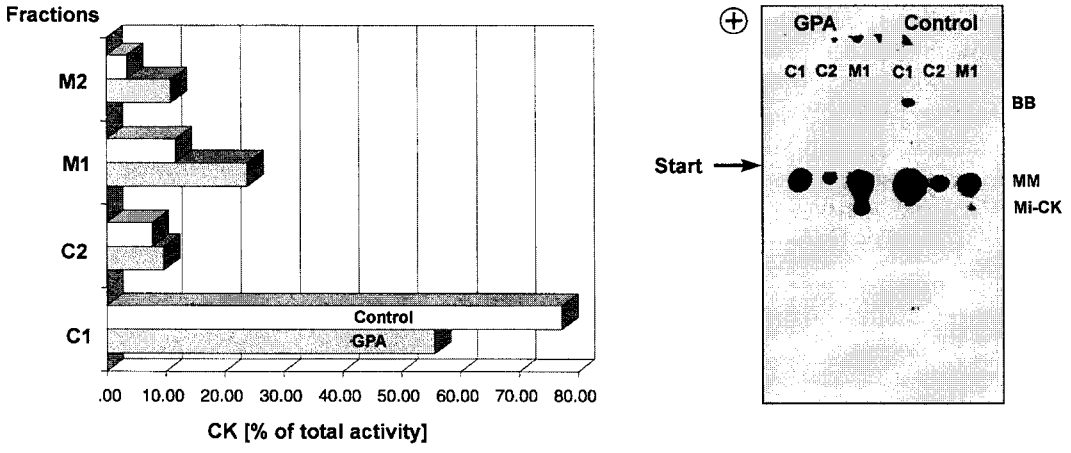

Fig. 3. Subcellular fractionation of creatine kinase isozymes in GPA and control soleus muscle. Representative figure showing soleus muscle from GPA-treated and control animals which was minced in a micro grinder, 100 mg of the minced soleus were extracted in a step-wise fashion, as described in Section 2. The extracts C1 and C2 contained mainly cytosolic enzymes, as seen from a high concentration of lactate dehydrogenase compared to very low concentrations of citrate synthase, representing a mitochondrial matrix protein marker (LDH/CS quotient in C1 of GPA soleus = 30.8, that of control = 31.5). Fractions M1 and M2 contained mainly mitochondrial enzymes as indicated by the low activity of lactate dehydrogenase compared to high citrate synthase activity (LDH/CS quotient in M1 of GPA soleus = 1.0, control value = 1.1). Left panel shows total CK activity determined in the different fractions by optical test. Right panel depicts the CK isozyme pattern of the different fractions. The mitochondrial fraction M1 from soleus of GPA-treated rats contains higher CK activity due to accumulation or overexpression of Mib-CK than that of control muscle (M1 of control lanes).

Table 1
Specific activities creatine kinase, adenylate kinase, hexokinase and succinate dehydrogenase in different tissues of control and GPA-treated animals

	CK U/g fw $n=6$	ADK U/g fw $n=3$	HK U/g fw $n=5$	SDH U/g fw $n=8$
Heart Control	47 ± 8	34 ± 3	3 ± 0.3	10 ± 4
GPA	28.4 ± 5 (−40%)	40 ± 16 (n.s.)	1.4 ± 0.4 (−54%)	7 ± 2 (−33%)
Brain Control	7 ± 0.5	17 ± 5	–	1.5 ± 0.1
GPA	11 ± 4 (+36%)	35 ± 1 (+100%)	–	3 ± 1 (+100%)
Soleus Control	45 ± 3	20 ± 6	1 ± 0.8	1.3 ± 0.5
GPA	27 ± 17 (−40%)	53 ± 5 (+165%)	0.63 ± 0.4 (−37%)	3.4 ± 1.6 (+160%)

Specific enzyme activities of creatine kinase, hexokinase and adenylate kinase of heart, brain and soleus homogenates. All values are enzyme units of activity based on grams of tissue fresh weight. The percentage values are given as the approximate mean increase or decrease in activity of the particular enzyme in the different GPA rat tissues relative to control (n.s., not significant).

A completely different picture emerges in GPA brain where total CK specific activity more than doubles when compared to control, which is mainly due to over-expression of cytoplasmic B-CK (Table 2), as Mia-CK increases only by approx. 50%.

These results clearly indicate that creatine depletion affects the expression of CK isoenzymes in a tissue-specific manner, and may explain why Mib-CK-rich inter-mitochondrial inclusion bodies are only seen in skeletal muscle, to a much lesser extent in heart and not at all in brain (O'Gorman et al., unpublished).

3.4. Function of mitochondrial creatine kinase in skinned soleus muscle fibres after chronic GPA feeding

Experiments with isolated mitochondria from normal muscle showed the regulation of oxidative phosphorylation through Mi-CK activity by providing ADP via stimulation with creatine [30,31]. We studied this role of Mi-CK in regulation of oxidative phosphorylation after chronic GPA treatment. Respiration of skinned soleus fibres, that are permeable to external substrates, was followed by oxygen electrode as a means to monitor activity of OXPHOS (Table 3). Under conditions saturating OXPHOS with phosphate, substrate and oxygen, addition of 0.1 mM ADP was not sufficient to fully activate respiration. In both groups of GPA-treated and control rats 76% of maximal state 3 (as seen with 2.0 mM ADP) was observed. Considering that inhibition of OXPHOS by atractyloside decreased the total respiration only by 50%, addition of 100 μM ADP therefore produced half of the possible activation. However, complete activation of OXPHOS was achieved in the controls at this ADP concentration through Mib-CK activity (in the presence of 10 mM Cr) [32]. In contrast, this was not possible in solei from GPA-treated rats. In this case Mib-CK could not be activated by creatine and the deficit in stimulation of oxidative phosphorylation persisted. Thus, a clear difference in creatine-stimulated oxidative phosphorylation between soleus muscle of normal and GPA-fed animals was observed. This indicates that in the latter case, even though the Mib-CK amount is increased, the enzyme is incapable of mediating creatine stimulation of OXPHOS.

Table 2
Specific CK activity in total tissue homogenates and isolated mitochondria from brain and heart of control and GPA-fed rats

mU/mg	(1) GPA Brain	(2) %	(3) GPA Heart	(4) %	(5) Control Brain	(6) Control Heart
Homogenate	217 ± 50	233	235.8 ± 73	75	93 ± 14	313.4 ± 14
Mitochondria	83 ± 2.0	154	112 ± 25	76	54 ± 13	148 ± 31

Specific activity of creatine kinase in homogenates and isolated mitochondria from brain and heart of control and GPA-fed rats. The percentage values shown in columns 2 and 4 are based upon the respective control activities in columns 5 and 6 as 100%, activities are expressed as mU/mg protein ($n=4$).

3.5. Isozymes of creatine kinase in brain and heart after chronic GPA treatment

Chronic feeding with GPA does not affect all tissues in the same way as was described for soleus. Mitochondrial inclusions typical for skeletal muscles [33] were not observed after 4 months feeding in heart and brain mitochon-

Table 3
Respiration analyses of Cr and non Cr-stimulated OXPHOS of skinned soleus fibres from GPA and control-fed rats

Additions	GPA % of state 3 max	Control % of state 3 max
0.1 mM ADP	76% (±7)	76% (±9)
2.0 mM ADP	100%	100%
0.1 mM Atract	54% (±9)	55% (±9)
10 mM Creatine		
0.1 mM ADP	72% (±16)	99% (±4)
2.0 mM ADP	92% (±2)	101% (±1)
0.1 mM Atract	46% (±11)	55% (±16)

Succinate dependent respiration analysis data of skinned soleus fibres (15 mg) from GPA- and control-fed rats. Stimulation of OXPHOS was carried out in the presence of 0.1 mM ADP firstly, followed by 2.0 mM ADP in the presence or absence of 10 mM Cr. 100% state 3 was taken as the oxygen consumption in the presence of 2.0 mM ADP for both GPA and control solei fibres, 0.1 mM atractyloside was then added to inhibit ADP-dependent OXPHOS. Other percentage values are the percentages relative to maximum state 3 values ($n=6$).

dria. The latter observation can be explained, as it is known that certain cells in the brain can synthesise creatine themselves [34]. We thus assumed that the brain could serve as an internal control in the experiments. However, unexpectedly the total activity of creatine kinase increased in brain homogenates by more than two-fold after GPA feeding (Table 2). The increase was mainly due to induction of the cytosolic BB-CK isozyme, as was concluded from the smaller activity change in the mitochondrial fraction. In contrast, the creatine kinase activity of heart muscle decreased 25% during GPA treatment, both in the crude homogenate as well as in the mitochondrial fraction, suggesting that it was mainly the mitochondrial fraction that lost CK activity.

3.6. Effects of chronic GPA treatment on isolated mitochondria from heart and brain

We monitored, by respirometry, the regulation of OXPHOS through Mi-CK also in isolated mitochondria (Table 4). When ADP was added, at a final concentration of 100 μM, to isolated mitochondria from control and GPA rat hearts, it was too low in both cases to fully activate oxidative phosphorylation. Complete activation of oxidative phosphorylation was observed, however, in the presence of 2 mM ADP. Control of oxidative phosphorylation through Mib-CK was then studied in the presence of 15 mM creatine by the addition of 0.5 or 5 mM ATP. Under these conditions the activity of oxidative phosphorylation depends completely on the rate of ADP synthesis via Mi-CK. By applying this test, almost 100% activation was observed by Mia-CK in brain mitochondria from GPA-treated rats (Table 4). In contrast, the mediation of creatine stimulation by Mib-CK in heart mitochondria from GPA treated rats was reduced at both ATP concentrations. Furthermore, we investigated whether hexokinase bound to the mitochondrial surface was able to stimulate oxidative phosphorylation in isolated heart and brain mitochondria from control and GPA-fed animals. The experiments were performed in the presence of pyruvate kinase/phosphoenolpyruvate as a scavenger system for free ADP. During isolation of brain mitochondria, almost all hexokinase activity remains bound to the mitochondria, in contrast to heart muscle, where mitochondria lose most of the activity. This might explain why OXPHOS was activated via hexokinase activity in brain mitochondria in contrast to heart mitochondria, no matter whether mitochondria from GPA-treated or control animals were investigated. However, in any case the stimulation of OXPHOS via hexokinase was indeed less for GPA heart mitochondria than for control. This may be due to the fact that there was a 50% decrease in the total activity of this enzyme in GPA heart tissue (Table 1).

3.7. Changes in other mitochondrial kinases and inner membrane enzymes

Besides Mi-CK, other kinases at the mitochondrial periphery, such as hexokinase and adenylate kinase, are thought to be involved in regulation of OXPHOS [35–37]. Thus, an increase in their activities may be expected in order to compensate for the missing Mi-CK function in

Table 4
Stimulation of OXPHOS by ADP, ATP + creatine, and ATP + glucose of isolated brain and heart mitochondria from control and GPA-treated rats

	GPA				Control			
	Brain		Heart		Brain		Heart	
ADP/O	1.6		1.7		1.65		1.65	
Additions	nmol O_2/ min × mg	% State 3	nmol O_2/ min × mg	% State 3	nmol O_2/ min × mg	% State 3	nmol O_2/ min × mg	% State 3
0.1 mM ADP	30.7 ± 6.6	81.1	41.69 ± 2.3	90.66	25.74 ± 4.1	83.7	35.0 ± 9.3	100.4
2.0 mM ADP	37.91 ± 7.4	100	45.98 ± 0.7	100	30.75 ± 5.4	100	34.89 ± 8.8	100
0.5 mM ATR	10.65 ± 1.4	28.1	10.83 ± 1.6	23.56	7.38 ± 0.3	24.0	9.62 ± 2.3	27.6
15 mM Creatine								
0.5 mM ATP	30.1 ± 5.4	79.3	17.67 ± 1.8	38.4	23.4 ± 2.9	76.1	39.1 ± 9.1	112
5 mM ATP	33.6 ± 3.5	88.6	32.57 ± 1.6	70.8	27.0 ± 1.5	88.2	40.43 ± 8.7	115.9
0.5 mM ATR	11.7 ± 0.5	30.9	7.1 ± 0.3	15.4	8.33 ± 0.8	27.1	10.57 ± 3.2	30.3
PK/PEP								
2 mM Glucose	12.25 ± 1.7	32.3	11.85 ± 1.4	25.8	10.49 ± 0.8	34.1	11.24 ± 3.0	32.2
5 mM ATP	31.97 ± 2.9	84.3	26.45 ± 3.0	57.5	26.4 ± 3.1	85.9	22.97 ± 7.6	65.8
0.5 mM ATR	11.4 ± 1.0	30.1	11.14 ± 2.6	24.23	8.25 ± 1.2	26.8	9.3 ± 2.3	26.7

Stimulation of succinate-dependent oxidative phosphorylation in isolated mitochondria from heart and brain of GPA-treated rats. The oxidation rate of approx. 1 mg of mitochondria was measured with an oxygen electrode in isolation medium in the presence of 5 mM succinate, 5 mM $MgCl_2$ and 5 mM phosphate. State 3 respiration was initiated by addition of either ADP or ATP as indicated. State 4 respiration was analysed after addition of 0.5 mM of atractyloside (ATR). In experiments analysing the function of hexokinase 2 IU pyruvate kinase (PK), 1 mM phosphoenol pyruvate (PEP) and 2 mM glucose was present. Hexokinase activity in the mitochondrial fraction of brain was 100 mU/mg (GPA) and 200 mU/mg (control) while heart mitochondria contained 2–5 mU/mg (GPA) and 5–10 mU/mg (control).

tissues from GPA-treated rats. We observed a significant increase after GPA treatment in total adenylate kinase activity of brain and soleus muscle, but not in heart, whereas in contrast, hexokinase activity decreased in heart and soleus (Table 1). Succinate dehydrogenase, representing a mitochondrial inner membrane marker, increased in brain and soleus by about 2-fold and the heart showed a slight decrease in activity (Table 1).

All the above results again show that there are many different tissue-specific metabolic adaptations to Cr depletion that may help maintain essential cellular processes to occur at normal rates.

4. Discussion

4.1. Effect of creatine depletion on creatine kinase isoenzyme expression in different tissues

Long-term GPA feeding gives rise to mitochondrial inclusion bodies which are also seen regularly in muscle biopsy material taken from mitochondrial myopathy patients [12,38]. A major difference between the inclusions seen in GPA-fed rats and those seen in human material, is that the type 1 crystals, between inner and outer membrane, as described by Farrants et al. [39], are rarely observed. Since the human and rat mitochondrial inclusion bodies are enriched with Mib-CK [12,38], we conclude that these are the same lesions.

Compared to the cytosolic isozymes, the amount of Mib-CK, both in enzyme activity and amount of protein present, increased after chronic GPA treatment (Fig. 2 and see Section 3). This is in direct contrast to a paper recently published, which theorized rather strongly against any increase of Mi-CK due to Cr depletion [40]. The approximate 4-fold increase in Mi-CK of GPA soleus reported here is accompanied by an observed decrease in the CK cytosolic isoform, as seen with the fractional extraction experiment (Fig. 3). It is unclear whether or not this is a regulatory event occurring at the transcriptional or translational level for both isoforms. By contrast, in brain of GPA-treated animals, where the substrate is intrinsically synthesised [34], the cytosolic enzyme was found to be induced (Tables 1 and 2).

4.2. Function of Mi-CK in soleus and heart of GPA-fed rats

To test whether this increased amount of Mi-CK is functionally active, OXPHOS stimulation was performed with skinned soleus fibres from control and GPA-fed rats (Table 3). We observed that in both cases addition of 0.1 mM ADP alone was not sufficient to fully activate OXPHOS, most likely because of diffusion limitations of ADP, since the effective ADP concentration may have been somewhat lower inside the fibres. However, this concentration (100 μM) represents the highest ADP level that is physiologically observed in active muscle [35]. With control solei, full activation of OXPHOS was achieved with 0.1 mM ADP in the presence of 10 mM Cr [32]. The excess Mib-CK in solei from GPA-treated rats was inactive in this test, indicating that dense packaging of Mib-CK in the intermembrane spaces may prevent access of ATP and Cr to the active sites of the enzyme. It would therefore be interesting to test whether the Mi-CK in muscle biopsy material, from patients suffering from mitochondrial myopathies, is similarly inactive.

The same effect was seen in mitochondria isolated from the hearts of the same GPA-fed rats. As no inclusion bodies were seen in these cardiac mitochondria, it is very difficult to explain this result. The specific activity of Mib-CK was shown not to increase, but if anything, to decrease slightly in these mitochondria when compared to control (Table 2). A similar finding was reported by Clark et al. [41].

On the whole, our data clearly demonstrate the role of Mi-CK in saturating the ANT and OXPHOS at low ADP concentrations, supporting the work of Saks et al. [32] and that this function is lost in GPA-fed rat cardiac and soleus mitochondria.

4.3. Compensation of missing creatine kinase activity

To avoid large ATP/ADP fluctuations during fast and fluctuating energy consuming processes, Cr/PCr is used as an energy buffer and energy transferring system. Obviously, the question of how this loss of creatine kinase function can be compensated for in GPA-treated animals, arises. Subtle effects have been found in the decreased twitch force and extended length of relaxation time of stimulated creatine depleted skeletal muscle [42]. The compensations that must arise to account for these subtle changes are now discussed. Firstly, it was found in heart muscle of GPA-treated rats that the level of free ADP is increased [18]. Secondly, an increase in mitochondrial aerobic capacity, especially in fast glycolytic fibres was described [19], as well as an increase of V_{max} of mitochondrial ATP-synthase [43]. Another report describes the more aerobic character of skeletal muscle physiology depleted of creatine [40], which is in line with our observation of increased succinate dehydrogenase activity (a marker for inner mitochondrial membrane) in soleus and brain tissue of GPA rats (Table 1). As a third possibility, we presumed that other kinases, such as hexokinase and adenylate kinase, which are organised at the mitochondrial peripheral surface and directly communicate with the inner mitochondrial compartment [37], may be induced.

The function of hexokinase in stimulation of OXPHOS was clearly demonstrated in brain mitochondria (Table 4). In this assay, we obtained full activation of the oxidative phosphorylation through the activity of hexokinase such as was seen with creatine kinase in brain mitochondria from

control and GPA-treated animals. These experiments furthermore indicated a direct communication between hexokinase and the inner mitochondrial compartment as they were performed in the presence of added pyruvate kinase and phosphoenol pyruvate, trapping free ADP. This was not seen in isolated heart mitochondria. While total hexokinase activity was decreased in cardiac and soleus muscles of GPA-treated animals, adenylate kinase activity was significantly increased in both brain and soleus of the same animals (Table 1). Comparable to CK, two isozymes of adenylate kinase exist, one being cytoplasmic and the other mitochondrial, and an interplay between the two adenylate kinases may provide an energy-shuttling system similar to that proposed for CK [44,45]. The importance of this increase of adenylate kinase in GPA muscle cells is more clearly understood in the light of the fact that a 58% decrease in AMP deaminase also occurs in rat soleus muscle after 6 weeks GPA feeding [21]. Thus, an increase in AMP follows an increase of ADP, via adenylate kinase in the cytoplasm, which can then be used by mitochondrial adenylate kinase in the intermembranous space to stimulate OXPHOS. Another important compensatory mechanism is also seen in the induction of ANT. It is conceivable that by this mechanism the ADP binding sites are increased as a reaction to decreased ADP supply through Mi-CK, due to its inactivity. All these compensatory mechanisms may explain why, in a recent report, creatine-depleted rats showed only subtle changes in exercise tolerance. The effect of GPA supplementation upon the rat physiology was reportedly similar to that of long endurance training [46]. Our results and those presented from null-mutants of transgenic mice that no longer express MM-CK [47], emphasise the ability of muscle cells to compensate for a compromised PCr/CK backup energy supply system.

Finally, our data imply that upon disruption of the PCr/Cr circuit, tissue-specific changes occur in the expression of enzymes and/or isoenzymes involved in cellular energy homeostasis, with the different tissues responding by emphasising specific metabolic pathways.

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