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Stimulation of Mitochondrial Gene Expression and Proliferation of Mitochondria Following Impairment of Cellular Energy Transfer by Inhibition of the Phosphocreatine Circuit in Rat Hearts

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Mitochondria proliferate when cellular energy demand increases. However, the pathways leading to enhanced expression of mitochondrial genes are largely unknown. We tested the hypothesis that an altered flux through energy metabolism is the key regulatory event by decreasing mitochondrial energy supply to rat heart cells by creatine depletion. Electron microscopy showed that the density of mitochondria increased by 75% in such hearts ($p < 0.01$). Levels of representative mRNAs encoded on mitochondrial DNA (mtDNA) or on nuclear chromosomes were elevated 1.5 to 2-fold ($p < 0.05$), while the mtDNA content was unchanged. The mRNA for the nuclear encoded mitochondrial transcription factor A (mtTFA) was increased after GPA feeding ($p < 0.05$). Thus, we have shown that an impairment of mitochondrial energy supply causes stimulation of gene expression resulting in mitochondrial proliferation, probably as a compensatory mechanism. The observed activation of the mtTFA gene corroborates the important function of this protein in nuclear–mitochondrial communication.

KEY WORDS: Mitochondrial biogenesis; energy metabolism; copy number; creatine kinase; mitochondrial transcription factor; nuclear–mitochondrial communication; high-mobility group proteins.

INTRODUCTION

Mitochondria produce ATP by oxidative phosphorylation (OXPHOS), providing most of the energy for ATP-consuming processes of the cell. In order to satisfy differing energy demands, different cell types of an organism are equipped with varying amounts of mitochondria. In many cells and tissues, the biogenesis of mitochondria can be stimulated when the energy demand is increased, leading to an enhanced capacity for OXPHOS. Well-known examples are the increased synthesis of mitochondrial enzymes in skeletal muscle after endurance training (Pette and Vrbova, 1992) or the proliferation of mitochondria in liver, heart, and skeletal muscle accompanying the increase of standard metabolic rate in the hyperthyroid state (Rolfe and Brown, 1997). Under these conditions, the genes for mitochondrial proteins, which are encoded either on nuclear chromosomes or by the small mitochondrial genome, are almost invariably upregulated (for a recent review, see Wiesner, 1997). However, the intracellular signals and possible transcription factors leading to such a stimulation of transcription, and thus to overall mitochondrial proliferation, are largely unknown. Changes

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in the ATP/ADP system were proposed to be a key event in skeletal muscle transformation during endurance training (Green *et al.*, 1992). However, recently it has been shown that elevated levels of intracellular calcium could also be a signal for the muscle fiber transformation observed under such conditions (Kubis *et al.*, 1997). In the hyperthyroid state, it is not clear whether increased hormone levels induce mitochondrial biogenesis per se or whether the increase of the standard metabolic rate, mostly due to activation of the Na^+/K^+-ATPase by thyroid hormone (Ismail-Beigi, 1993), is the primary key stimulus.

In order to test the hypothesis that disturbances of energy metabolism are the key regulatory event, we reasoned that a decrease of cellular energy supply should result in metabolic signals similar to those induced by increasing cellular energy demand and, consequently, upregulation of mitochondrial genes. Therefore, expression of mitochondrial genes was analyzed in various tissues of rats, which were fed the creatine analog β-guanidinopropionic acid (GPA), which selectively disturbs intracellular transfer of high-energy phosphate from mitochondria to energy-consuming sites by the creatine/phosphocreatine circuit (Wallimann *et. al.*, 1992). The creatine depletion induced by this treatment is known to induce, beside a host of metabolic adaptations (Wyss and Wallimann, 1994), also an accumulation of abnormally large mitochondria in skeletal muscle, which are, however, restricted to the subsarcolemmal space. After GPA feeding, these enlarged subsarcolemmal mitochondria, with crystalline inclusions of mitochondrial creatine kinase (Mi-CK), appear first in soleus muscle and later on also in other muscle types as well (O'Gorman *et al.*, 1996). Here, the cardiac ventricle was chosen for a detailed investigation of (1) the expression of nuclear genes encoding for mitochondrial proteins, (2) the abundance and expression of mitochondrial DNA, and (3) organelle density. Finally, the expression of mitochondrial transcription factor A (mtTFA) was also analyzed in a quantitative manner in GPA-fed versus control animals. MtTFA, a DNA binding protein, encoded in the nucleus and targeted to mitochondria, is likely to be involved in regulation of transcription as well as in replication of mtDNA (Shadel and Clayton, 1997) and thus represents a good candidate for transmitting signals generated in the cytosol to mtDNA.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats were either kept on control diet or on a diet containing 2.5% GPA (weight/weight) and an additional 1% GPA (weight/vol.) in the drinking water. This treatment was started at an age of 3 weeks and proceeded for an additional 6 weeks. Rats were killed under quick anesthesia with CO_2, tissues were dissected, and immediately frozen in liquid nitrogen.

Isolation and Blotting of Nucleic Acids

Total RNA was isolated from tissue pulverized under liquid nitrogen according to Chomczynski and Sacchi (1987). The final RNA pellet was dissolved in water and the RNA concentration was quantitated by measuring the absorbance at 260 nm. Steady-state levels of nuclear and mitochondrial mRNAs were determined by slot blot analysis. The integrity of mRNA was first checked in 1.0% agarose gels stained with ethidium bromide (Sambrook *et al.*, 1989). RNA samples were then dissolved in 100 μl of water, denatured at 70°C for 15 min with 300 μl of 18% formaldehyde/10× SSC, and blotted onto nylon membranes with a slot blot filtration device in serial dilutions (5, 2.5, and 1.25 μg for nuclear transcripts; 0.6, 0.3, and 0.15 μg for mitochondrial transcripts). Northern blots were prepared using formaldehyde-containing agarose gels, nitrocellulose, and the capillary transfer method (Sambrook *et al.*, 1989). In order to measure mtTFA mRNA, 25 μg of total RNA was loaded onto the gel.

Genomic DNA was extracted from ventricle powder by proteinase K digestion and phenol/chloroform extraction (Sambrook *et al.*, 1989). For quantitation of mitochondrial DNA (mtDNA), genomic DNA was digested with *XbaI*/*XhoI*, which linearizes rat mtDNA, denatured by acid treatment, neutralized, and blotted onto nitrocellulose membranes with a slot blot device (dilution series 1, 0.5, and 0.25 μg).

Hybridization of DNA and RNA Blots

Blots were prehybridized for 2 h and hybridized overnight at 42°C (prehybridization: 40% formamide, 5× SSC, 50 mmol/L phosphate buffer, pH 7.4, 10 × Denhardts solution, 0.2% SDS, 500 μg/ml salmon sperm DNA; hybridization: 50% formamide, 3 × SSC, 10 mmol/L phosphate buffer, pH 7.4, 2 × Denhardts solution, 0.2% SDS, 170 μg/ml salmon sperm DNA). cDNA probes isolated from appropriate plasmids or PCR products labeled to high specific radioactivity by the random priming method were used (Sambrook *et*

al., 1989). After hybridization, blots were washed at 42°C (2 × 15 min in 2× SSC, 0.1% SDS followed by 2× 15 min in 0.1× SSC, 0.1% SDS). Between hybridizations, blots were stripped from the previous probe (4 × 5 min incubations in boiling 0.01× SSC, 0.01% SDS) and were finally hybridized to a probe for cytosolic 28 S rRNA. For this probe, hybridization temperature was 44°C and the last two washing steps were performed at 50°C. Probes and hybridization conditions were described in detail previously (Garstka *et al.*, 1994; Wiesner *et al.*, 1992a). In addition, a 1900-bp probe for rat cytochrome *c* cloned into the *KpnI–BamHI* sites of the plasmid RC4-4B (Virbasius and Scarpulla, 1990) and a 800-bp probe for GAPDH (generous gift of Dr. Schlueter, Giessen) was used under the same conditions. In order to measure mtTFA mRNA, a 1550-bp probe encoding mouse mTFA cloned into the *EcoRI* site of pBS-KS was used (Garman and Clayton, unpublished); in this case, hybridization temperature was 38°C, yeast t-tRNA was used instead of salmon sperm DNA for blocking, and only two washes were employed after hybridization (2 × 15 min, 0.1× SSC, 0.1% SDS, 38°C). Blots were exposed to X-ray films, and the films were evaluated densitometrically using a video camera-based analysis system and the AIDA, Version 1.0, software (Raytest). For normalization, densitometric data for transcripts and mtDNA were normalized to the 28 S rRNA signal, taking care that the signal was in the linear range. Values obtained for control hearts were set to 100 arbitrary densitomeric units.

Immunoblotting

Small pieces of frozen tissue were homogenized in boiling 62.5 m*M* Tris (pH 6.8), 2% SDS, 10% glycerol using a small glass-teflon homogenizer. For the determination of cytochrome *c* oxidase levels, equal amounts of protein as measured by the method of Bradford (1976) were loaded on slab gels (15 × 15 × 0.15 cm) with a 12–15% polyacrylamide gradient and a 3% stacking gel. Gels were prepared and run according to the method of Laemmli (1970) at 60 V, 20 mA for 16–18 h at room temperature. For determination of mtTFA levels, protein samples were run on 12.5% slab gels (8 × 7 × 0.15 cm) and a 3% stacking gel at 100 V, 15 mA for 3 h.

Proteins were transferred to nitrocellulose in an electroblot apparatus in 154 m*M* glycine, 20 m*M* Tris (pH 8.3), and 20% methanol at 30 V, 100 mA for 3 h. Blots were subsequently blocked in 20 m*M* Tris, pH 7.5, 150 m*M* NaCl, 0.5% TWEEN, 2% bovine serine albumin, and 1% milk powder for 2 h and incubated overnight in the same buffer containing rabbit antiserum raised against human cytochrome *c* oxidase (CO) or against recombinant human mtTFA. After washing, protein bands were visualized by incubation with goat-antirabbit IgG antiserum, horse radish-peroxidase conjugated, and chemiluminescence detection (ECL, Amersham). Chemiluminescent blots were exposed to X-ray films and bands of interest were evaluated densitometrically using a video camera-based analysis system and the AIDA, Version 1.0, software (Raytest).

Determination of CO Activity

For analysis of cytochrome *c* oxidase (CO) activity as a marker of OXPHOS capacity, a small piece of frozen muscle was weighed (20–30 mg) and homogenized with a glass homogenizer and pestle in 1 ml of ice-cold phosphate buffer (100 m*M*, pH 7.0). Maximal enzyme activity was determined spectrophotometrically by measuring the rate of oxidation of reduced horse heart cytochrome *c* (Sigma), reflected by the change in absorbance at 550 nm (Cooperstein and Lazarow, 1951). The protein concentration of the homogenates was measured by the method of Bradford (1976) using BSA as standard. Enzyme activity was then expressed as enzymatic units (μmol cytochrome *c* min^{-1} mg $protein^{-1}$), using the millimolar extinction coefficient of 29.5 for reduced horse heart cytochrome *c*.

Electron Microscopy

Samples from left ventricles 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 containing 0.15 *M* sucrose, and postfixed 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 ultrathin sections were cut (Ultracut E., Reichert/Jung). The sections were observed on carbon-coated grids after staining with uranyl acetate and lead citrate, using a transmission

electron microscope (Jeol 100C). Micrographs were taken at 80 kV and a magnification of 8.300-fold.

Mitochondria were counted on two randomly selected fields of similar areas from three individual ventricles per group by three different investigators who did not know to which experimental group the individual micrographs belonged. Mitochondrial profiles were unequivocally identified as vesicles of appropriate size and the clear presence of internal, cristaelike structures.

Statistical Evaluation of Results

Results are expressed as mean values ± SD and groups were compared by Students *t*-test; $p < 0.05$ was assumed to be statistically significant.

RESULTS

In a first screening, a Northern blot of RNA from different rat tissues showed that the mRNA for cytochrome *c* oxidase (CO) subunit III, standardized against 28 S rRNA, was markedly enhanced in heart and marginally elevated in soleus muscle after 6 weeks of GPA feeding, while there was no obvious difference compared to controls in quadriceps muscle or brain (Fig. 1). The ventricle was chosen for further investigation. Electron microscopy showed that a significant proliferation of mitochondria occurred in left ventricles after GPA feeding (Fig. 2). Quantitation showed that the density of organelle profiles was markedly increased by about 75% (42 ± 11 profiles per field in controls versus 74 ± 23 in GPA treated animals, $p < 0.01$). Mitochondria were regularly interspersed between myofibrils and, except for the higher density, no obvious difference compared to the cytoarchitecture of control hearts was observed. However, individual mitochondria were clearly smaller in ventricles of GPA-treated rats and, in some but not all organelles, the inner membrane system seemed to be disorganized when compared to control animals.

Myocardial levels of three transcripts encoded on mtDNA, as well as three transcripts for mitochondial proteins encoded on nuclear chromosomes, were measured. A significant increase was found for the mito-

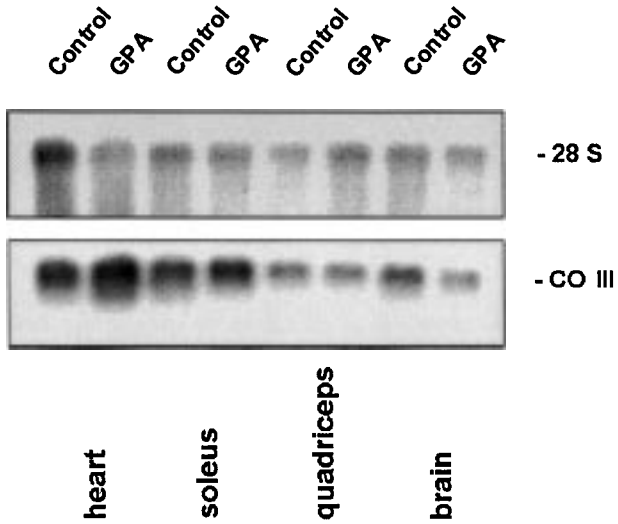

Fig. 1. Northern blot analysis of mitochondrial mRNA encoding cytochrome *c* oxidase subunit III (CO III) in four different tissues taken from GPA-treated and control rats. Also shown is the signal for the cytosolic 28 S rRNA used for normalization.

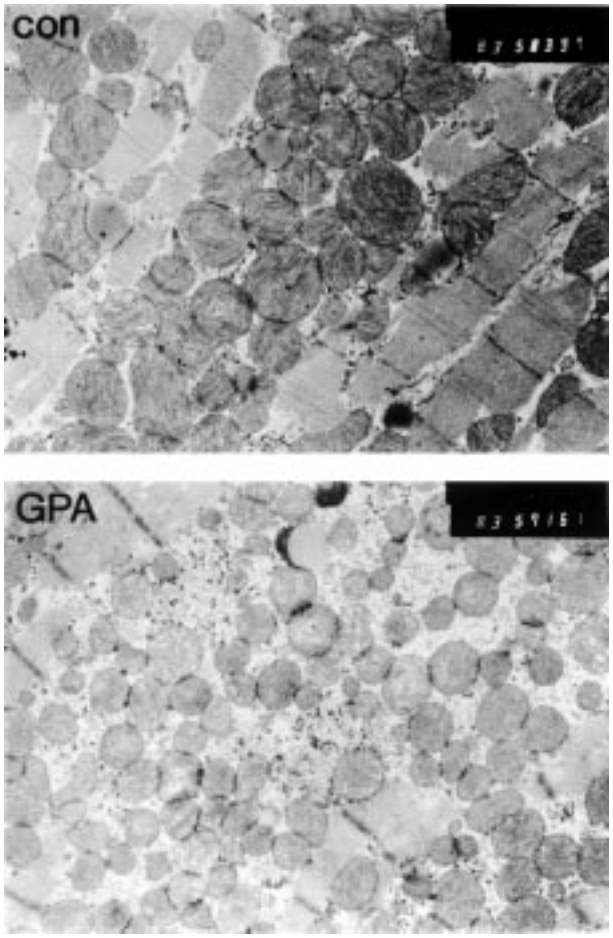

Fig. 2. Electron micrographs of left ventricles from control (upper panel) and GPA-fed rats (lower panel). Magnification was 8.300 ×.

chondrial mRNAs for CO subunits I and III, while no significant changes were observed for mitochondrial 12 S ribosomal RNA (Fig. 3A). The mRNAs for CO subunit Vb and VIc, as well as for the substrate of CO, cytochrome *c*, which are all encoded on nuclear chromosomes, were also significantly elevated (Fig. 3B). Slot blot analysis of ventricular DNA showed that the abundance of mitochondrial DNA was rather similar (densitometric values were 100 ± 18 versus 122 ± 18; control versus GPA-treated animals; p = n.s).

Interestingly, these changes were not associated with an increased content of CO on the protein level, shown by measurement of enzyme activity (0.27 ± 0.04 versus 0.26 ± 0.03 enzymatic units/mg protein; control versus GPA treated animals; p = n.s.) as well as Western blot analysis of individual subunits (Fig. 4). The antiserum, which was used, recognizes CO subunits I, II, and III, the latter two not being clearly separated when loading tissue homogenates. These are

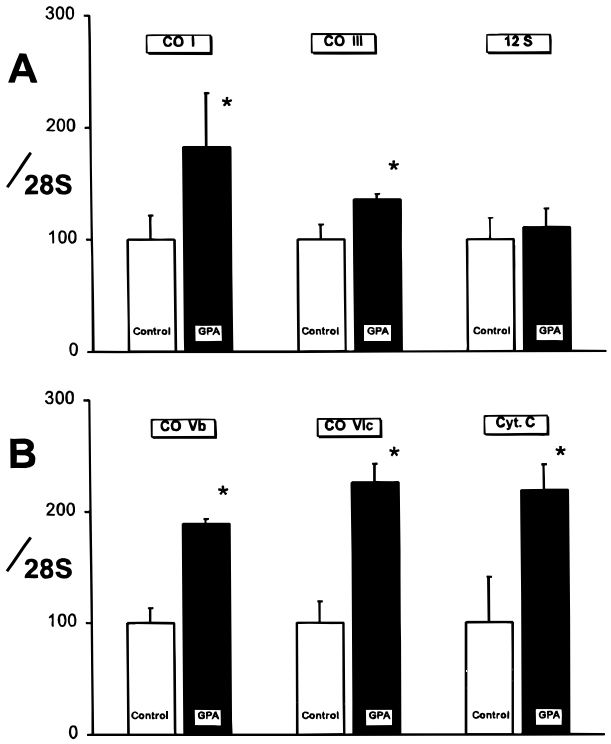

Fig. 3. Levels of transcripts encoded on (A) mitochondrial DNA and (B) nuclear chromosomes, normalized to cytosolic 28 S rRNA, in left ventricles from control (open bars) and GPA-treated rats (black bars). Values were obtained by slot blot hybridization analysis, autoradiography, and densitometry and care was taken that the signal was in the linear range for all measurements. *, Significantly different from control values ($p < 0.05$).

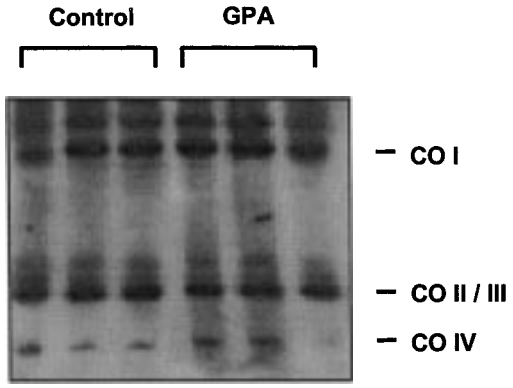

Fig. 4. Western blot analysis of cytochrome *c* oxidase subunits in ventricles of control or GPA-fed rats. The four subunits of the enzyme complex, which are recognized by the polyclonal antiserum, are indicated.

encoded by the mitochondrial genome, while subunit IV, which is also detected, is encoded by the nucleus. Since it was obvious that no differences between the two groups occurred, no further efforts were made to improve the resolution.

The low abundance mRNA for mtTFA was detectable only after blotting of large amounts of RNA and under low stringency conditions of hybridization and washing (Fig. 5A). However, the levels of mtTFA mRNA, when normalized to 28 S rRNA, were significantly increased by 52% after GPA feeding (Fig. 5B, $p < 0.05$). Since large amounts of RNA had been loaded on these blots, we felt that normalization to the high abundance 28 S rRNA may not be reliable. Thus, blots were also hybridized to a cDNA probe for GAPDH, which is often considered to be a housekeeping gene. However, we found that GAPDH mRNA was also increased by 42% after GPA feeding, when normalized to 28 S rRNA (Fig. 5A). Thus, we calculated ratios for mtTFA/GAPDH from Northern blots (Fig. 5A) and GAPDH/28S from slot blots, making sure that signals were in the linear range for both measurements. By multiplication of these numbers, we finally obtained values for mtTFA/28 S, which were again higher in hearts of GPA-fed animals (+56%; $p < 0.05$), in agreement with our initial finding. By Western blotting, however, we could not demonstrate a significant increase of the mature mtTFA protein in these hearts (Fig. 5C).

DISCUSSION

The working hypothesis of the present study was that lowering of the phosphocreatine (PCr) versus cre-

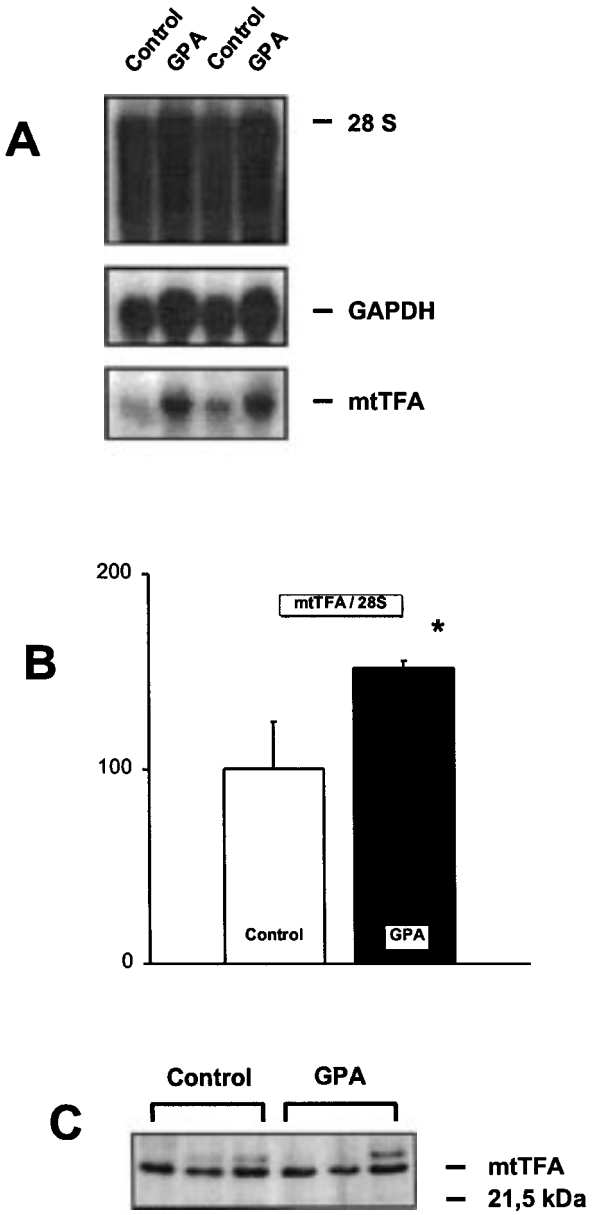

Fig. 5. Northern blot analysis of mtTFA gene expression in two representative ventricle samples per group of control or GPA-treated rats (A), quantitation of mtTFA mRNA levels (B), and Western blot analysis of mtTFA protein in three ventricles per group (C). In (A), the signals for GAPDH mRNA as well as 28 S rRNA, which were both used for normalization, are also shown (see text). *, Significantly different from control values ($p < 0.05$).

atine (Cr) ratio by GPA feeding and thus inhibiting the flow of ATP from mitochondria to energy-consuming sites via the phosphocreatine circuit would lead to an impairment of cellular energy homeostasis (Wallimann *et al.*, 1992), followed by increased transcription of mitochondrial genes leading to a compensatory mitochondrial proliferation in an attempt to restore the cellular energy status. This would support our hypothesis that metabolic signal(s) arising either from altered metabolite levels or from changing fluxes through energy-yielding pathways is/are the key stimulus for mitochondrial proliferation (Wiesner, 1997). Mitochondrial proliferation occurs under those physiological and pathological conditions where cellular energy demand increases. Well-known examples are endurance training of skeletal muscle (Pette and Vrbova, 1992), cold adaptation of brown adipose tissue (Klingenspor *et al.*, 1996), hyperthyroidism (Wiesner *et al.*, 1992a), or hyperglucocorticoidism (Van, 1992). Our results are in agreement with our working hypothesis, since a significant increase of organelle density was observed in the left ventricle of rats after depletion of PCr levels. Mitochondrial proliferation was accompanied by increased levels of mRNAs for CO subunits, encoded on nuclear chromosomes as well as mtDNA, but also by elevated mRNA for nuclear encoded cytochrome *c*. This corroborates previous observations that stimulated mitochondrial biogenesis in fully differentiated cells is regulated predominantly at the transcriptional level and that expression of nuclear genes and mtDNA is well coordinated on the long-term range (reviewed in Wiesner, 1997). In contrast, during differentiation of liver mitochondria in embryonic development, regulation of translational processes also occurs (Izquierdo *et al.*, 1995). No significant increase of 12 S rRNA, which probably reflects the abundance of mitochondrial ribosomes, was observed in our experiments. However, it should be noted that the pool of 12 S rRNA molecules is about tenfold higher in differentiated rat tissues when compared to any individual mitochondrial mRNA (Garstka *et al.*, 1994; Harting and Wiesner, 1997). Thus, a significant expansion of mitochondrial mRNA pools is more readily achieved when transcription of mtDNA is increased compared to rRNA pools.

Although the density of organelles almost doubled after GPA feeding, no significant upregulation of total mtDNA abundance was observed here. This indicates again that an increase of mtDNA copy number is (1) not a necessary prerequisite for mitochondrial proliferation, since the organelles contain several copies of the genome in most tissues and cells (Robin and Wong, 1988; Wiesner *et al.*, 1992b), and (2) is also not a key mechanism for increasing mitochondrial transcript levels via increased template availability (Wiesner, 1992).

Remarkably, the observed increase of mitochondrial density was also not accompanied by an increase of CO protein or CO activity, in accordance with our previous finding of unchanged succinate dehydrogenase acitivity in such hearts, another mitochondrial marker enzyme encoded exclusively by the nucleus (O'Gorman *et al.*, 1996). Taken together, inhibition of the phosphocreatine circuit selectively increases organelle number without a concomitant increase of the overall capacity for oxygen consumption. In terms of adaptation, this response is perfectly adjusted to the altered needs of the heart cell. It should be kept in mind that no increase of energy demand asking for a higher ATP-production rate had been induced by GPA feeding, but rather an inhibition of cellular energy transfer, asking for shorter diffusion distances between energy-producing (mitochondria) and energy-consuming sites. This is achieved by the compensatory increase of organelle density we observed. However, although mitochondrial proliferation can obviously occur without increasing the copy number of mtDNA, it is obviously obligatory to increase the abundance of mRNAs for mitochondrial proteins, those encoded on mtDNA as well as those encoded in the nucleus, in order to supply the increased number of organelles with sufficient protein.

The striking similarities between the changes induced by GPA in skeletal muscle and the hallmark of mitochondrial myopathies caused by mutations of mtDNA, so-called "ragged red fibers," have been previously discussed (Stadhouders *et al.*, 1994; Holtzman et al., 1997). Remarkably, a proliferation of mitochondria similar to our model was also reported recently in hearts of knockout mice deficient in the heart/muscle isoform of the adenine nucleotide translocator (Graham *et al.*, 1997), a new model for mitochondrial myopathy and cardiomyopathy. Unfortunately, no data on mitochondrial enzyme activities were reported for the ventricle in this work, but in skeletal muscle of these mice, CO as well as SDH increased (Graham *et al.*, 1997), as they do in skeletal muscle of GPA-fed rats (O'Gorman et al., 1996).

In summary, it seems clear now that any disturbancies of energy metabolism, whether caused by increases of energy demand or decreases of mitochondrial energy supply, causes mitochondrial proliferation as a compensatory mechanism. This is, of course, a futile cycle when nonfunctional mitochondria proliferate and even overgrow intact mitochondria, as is the case in ragged red fibers carrying mutated mtDNA during the progression of mitochondrial diseases in patients (Weber *et al.*, 1997). The nature of the initial metabolic signal remains unsolved to date, although a decrease of the ATP/ADP_{free} ratio during type II (fast-glycolytic) to type I (slow-oxidative) muscle fiber transformation (Green *et al.*, 1992) and an increase of ADP_{free} in hearts of GPA-fed rats (Mekhfi *et al.*, 1990) strongly suggest involvement of the adenylate system. This is supported by the fact that impairment of the CK system, either by feeding animals with GPA, or by ablation of the CK isoenzymes in muscles of transgenic animals (Steeghs *et al.*, 1997), both lead to an impaired cellular energy status with lowered PCr/Cr and ATP/ADP ratios, as well as increased levels of AMP (Wyss and Wallimann, 1994). An attractive mechanism involving AMP-activated protein kinase, acting as an energy sensor being regulated by AMP levels as well as by the PCr/Cr ratio (Ponticos *et al.*, 1998), may be suggested. This kinase, as a fuel gauge that recognizes ATP-depletion, plays an important role in the regulation of gene expression and fatty acid oxidation (Kemp *et al.*, 1999). In parallel with energy depletion caused by long-term GPA supplementation, a chronic Ca^{2+} overload can be expected, that has been verified in the CK knock-out animals, which display impaired muscle relaxation (Steeghs *et al.*, 1997). This is because of the fact that the most important function of the CK/PCr system is related to the highly demanding energetics of Ca^{2+} homeostasis (Wallimann *et al.*, 1998; Wallimann and Hemmer, 1994). Thus, elevated $[Ca^{2+}]$ as well as [AMP] are likely candidates to act as energy stress signals that induce adaptational changes, like mitochondrial proliferation in muscle (O'Gorman *et al.*, 1997).

More is known about possible pathways of communication between the cytosol, where the primary signal, Ca^{2+}, [AMP], and others, are probably being measured, and the mitochondrial compartment. Mitochondrial transcription factor A is a high-mobility group type, DNA binding protein, encoded by the nucleus and targeted to mitochondria. This protein, together with mitochondrial RNA polymerase, is sufficient to stimulate efficient and faithful transcription of mtDNA promoters in the test tube (Fisher *et al.*, 1989). In addition to a large body of information on this protein *in vitro* (reviewed in Shadel and Clayton, 1997), it has also been shown that mtTFA mRNA as well as mtTFA protein are upregulated by thyroid hormone in rat liver together with mitochondrial transcripts (Garstka *et al.*, 1994; Stein and Wiesner, unpublished), suggesting a causal relationship. This is greatly strengthened, on the one hand, by our data showing

that overexpression of mtTFA in HeLa cells and the import of this factor into isolated mitochondria increase the levels of mitochondrial transcripts and transcription rate, respectively (Montoya *et al.*, 1997; Weber, Wiesner et al., in preparation). On the other hand, mice carrying only one functional allele of the mtTFA gene (heterozygous knock-out) show an obviously gene-dosage dependent, reduced level of mtDNA in all tissues that were examined and also reduced levels of mitochondrial transcripts in the heart and kidney (Larsson *et al.*, 1998). Thus, it is clear that this protein plays an eminent role in regulation of mitochondrial transcription *in vivo*. However, although mitochondrial transcript levels were increased in hearts of GPA-fed rats (Fig. 3A), no increase of mtTFA protein was observed (Fig. 5C). The most likely explanation is that besides being a transcription factor interacting with sequences proximal to the two transcription initiation sites, mtTFA is also a high-abundance, high-affinity DNA-binding protein, covering an extended portion of the noncoding region of mtDNA. Interestingly, *in organello* footprinting has shown that the majority of mtDNA molecules in human placenta (Ghivizzani et al., 1994) as well as in rat liver (Cantatore et al., 1995) is covered with mtTFA at these latter sites. In contrast, the sites proximal to the two promoters are only partly occupied, thus being susceptible to regulation by changing levels of intramitochondrial mtTFA. Such changes, however, are expected to be too subtle to be detectable within the large total pool of mtTFA, which is displayed on Western blots. However, the significant increase of mtTFA mRNA (Fig. 5A and B) strongly suggests that disturbancies of the myocardial energy status, indeed, leads to activation of the mtTFA gene, corroborating the important function of this protein in nuclear–mitochondrial communication. Whether elevated Ca^{2+} and/or AMP levels, directly or indirectly affect the expression and function of mtTFA is unknown at present. However, a connection of these cellular factors to mitochondrial gene expression, possibly via mtTFA, is a likely and very attractive hypothesis to be tested.

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