

Regulation of mitochondrial gene expression by energy demand in neural cells

Zara Mehrabian,* Li-Ing Liu,† Gary Fiskum,* Stanley I. Rapoport† and Krish Chandrasekaran*

*Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, Maryland, USA

†Section on Brain Physiology and Metabolism, National Institute on Aging, National Institutes of Health, Bethesda, Maryland, USA

Abstract

Mitochondrial DNA (mtDNA) encodes critical subunit proteins of the oxidative phosphorylation (OXPHOS) complex that generates ATP. This study tested the hypothesis that mitochondrial gene expression in neural cells is regulated by energy demand, as modified via stimulation of cellular sodium transport. Exposure of PC12S cells to the sodium ionophore monensin (250 nM) for 1–6 h caused a 13–60% decrease in cellular ATP (from 15 to 5 nmol per mg protein at 6 h). Levels of mitochondrial DNA-encoded mRNAs (mt-mRNAs) increased significantly (150%) within the first hour of exposure to monensin, and then decreased significantly (50%) at 3–4 h. Levels of mtDNA-encoded 12S rRNA and nuclear DNA-encoded OXPHOS subunit mRNAs were not significantly affected. Exposure of primary cerebellar neuronal cultures to the excitatory amino acid glutamate caused a similar rapid and significant increase

followed by a significant decrease in cell mt-mRNA levels. The monensin-induced initial increase in mt-mRNA levels was abolished by pretreatment with actinomycin D or by reducing extracellular sodium ion concentration. The monensin-induced delayed reduction in mt-mRNA levels was accelerated in the presence of actinomycin D, and was accompanied by a 67% reduction in the half-life (from 3.6 to 1.2 h). Exposure of PC12S cells to 2-deoxy-D-glucose significantly decreased cellular ATP levels (from 14.2 to 7.1 nmol per mg protein at 8 h), and increased mt-mRNA levels. These results suggest a physiological transcriptional mechanism of regulation of mitochondrial gene expression by energy demand and a post-transcriptional regulation that is independent of energy status of the cell.

Keywords: mitochondrial DNA, monensin, mRNA half-life, post-transcription, RNase-L, transcription.

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The mammalian brain is characterized by a high rate of glucose consumption, high content of carriers and enzymes for glucose transport and metabolism, a high rate of oxidative phosphorylation (OXPHOS) in mitochondria, and a high ATP consumption (Erecinska and Silver 1989; Wong-Riley 1989). Furthermore, the rates of glucose utilization and activities of the OXPHOS enzymes that generate ATP correlate with neuronal activity (Hevner *et al.* 1995). ATP is the energy source for active ion pumping to maintain resting membrane potential, fast axoplasmic transport, membrane phospholipid turnover, synthesis of macromolecules and neurotransmitters, and other cellular processes (Erecinska and Silver 1989; Wong-Riley 1989; Purdon *et al.* 2002). Active ion pumping at synapses and dendrites by far consumes the most ATP (Erecinska and Silver 1989).

The mitochondrial respiratory chain responsible for generation of cellular ATP consists of five multisubunit OXPHOS enzyme complexes. Four of these, Complexes I, III, IV and V, are bipartite and consist of subunits derived from both mitochondrial DNA (mtDNA) and nuclear DNA

(nDNA). MtDNA encodes 13 polypeptides, all of which are necessary for electron transport and OXPHOS. The remaining subunits are specified by the nuclear genome. Both mtDNA and nDNA-encoded subunits are required to form active enzyme complexes (Attardi and Schatz 1988).

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Address correspondence and reprint requests to Dr Krish Chandrasekaran, Department of Anesthesiology, University of Maryland School of Medicine, Medical School Teaching Facility 5–34, 685 West Baltimore Street, Baltimore, MD 21201, USA.

E-mail: kchandra@anesthlab.umm.edu

Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium concentration; COX, cytochrome oxidase; cyt, cytochrome; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; mtDNA, mitochondrial DNA, mt-mRNA, mitochondrial DNA-encoded mRNA; $[Na^+]_e$, extracellular sodium concentration; $[Na^+]_i$, intracellular sodium concentration; ND, NADH dehydrogenase; nDNA, nuclear DNA; NRF, nuclear respiratory factor; OXPHOS, oxidative phosphorylation; SDH, succinate dehydrogenase.

Changes in levels of mtDNA-encoded mRNA (mt-mRNA) occur rapidly in response to changes in energy requirement of the cell. Decreased neuronal activity, induced by afferent impulse blockade, decreases neuronal mt-mRNA (Wong-Riley *et al.* 1997), whereas removal of the afferent impulse blockade restores basal mRNA levels (Hevner and Wong-Riley 1993), suggesting feedback regulation of mitochondrial gene expression by energy demand (Wong-Riley *et al.* 1997). Consistent with this reasoning, an *in organello* method demonstrated that high intramitochondrial ATP levels suppress transcription of mtDNA, suggesting how energy demand can regulate mtDNA transcription (Gaines and Attardi 1984; Enriquez *et al.* 1996; DasGupta *et al.* 2001). Apart from transcriptional control, mitochondrial gene expression depends on differences in RNA stability (Gelfand and Attardi 1981; Attardi *et al.* 1990). Although mitochondrial gene expression is a major component in the regulation of energy metabolism of the cell, the contributions of transcriptional and post-transcriptional mechanisms to this regulation are not known (Kagawa and Ohta 1990).

The aim of our present study was to investigate the mechanism(s) of regulation of mitochondrial gene expression under conditions of increasing energy demand. Cellular energy demand was increased by exposing rat pheochromocytoma PC12 cultures to the Na⁺ ionophore monensin (Pressman and Fahim 1982), or by exposing primary neuronal cultures to the excitatory amino acid glutamate (Ankarcona *et al.* 1995), then measuring ATP and levels of mtDNA-encoded and nDNA-encoded OXPHOS mRNAs. Our results indicate a selective initial increase and a subsequent decrease in mt-mRNA in both monensin- and glutamate-treated cells. The initial increase is due to transcriptional regulation dependent on the energy status of the cell, whereas the subsequent decrease is due to post-transcriptional regulation that is independent of the cell's energy status. Part of this work has been published as an abstract (Liu *et al.* 1999).

Materials and methods

Chemicals

Reagents and chemicals were of the highest grade available from Sigma Chemical Co. (St Louis, MO, USA). Stock solutions of ouabain and actinomycin D were prepared in water, whereas monensin and ionomycin were dissolved in 95% ethanol. When ethanol was used as a solvent, appropriate control experiments were conducted using the vehicle alone. Ethanol concentrations were always < 0.1%.

Culture of PC12S cells

A morphological variant of rat pheochromocytoma PC12 cells (PC12S) that has the ability to grow in tissue culture dishes without polylysine treatment was used in the experiments (Fukuyama *et al.* 1993). The PC12S cells were maintained in Dulbecco's modified

Eagle's medium (DMEM) containing 2 mM glutamine, 7.5% heat-inactivated fetal calf serum, 7.5% heat-inactivated horse serum and penicillin-streptomycin.

Primary rat cerebellar cultures

Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies (National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985).

Cerebellar granule neurons were prepared from brains of 7-day-old Sprague-Dawley rats using a standard method (Schousboe *et al.* 1989). Neurons were plated at a density of 2×10^5 cells/cm² in six-well tissue culture chambers coated with poly-L-lysine (MW 30 000–700 000) and were cultured in Eagle's basal medium supplemented with Earle's salts, 10% inactivated fetal calf serum, 25 mM KCl and gentamicin (50 ng/mL). Neuronal cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air. Twenty-four hours after plating, cytosine arabinoside (10 μM) was added to the cultures to prevent growth of glial cells. To ensure sensitivity to glutamate, we routinely used 8-day-old cerebellar neuronal cultures.

Exposure to monensin, ouabain, ionomycin or 2-deoxy-D-glucose

PC12S cells grown in 100 × 15 mm dishes were treated with vehicle, monensin (final concentration 250 nM), ouabain (final concentration 1 mM) or ionomycin (final concentration 3 μM). At timed points over a 6–8-h period, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium, and total RNA was isolated using RNA-Bee reagent as recommended by the manufacturer (TEL-TEST Inc., Friendswood, TX, USA). Total RNA was subjected to northern blot analysis as described below.

For experiments with 2-deoxy-D-glucose, this reagent was added at a concentration of 4.5 g/L to glucose-free DMEM containing pyruvate (110 mg/L). At timed points over an 8-h period, the cells were exposed to this medium and total RNA was isolated.

The effect of extracellular sodium ([Na⁺]_e) on monensin-induced changes in mt-mRNA levels was studied by changing [Na⁺]_e from 125 mM to 62.5 mM or 31.25 mM. Briefly, the amount of sodium chloride in constituted culture medium was decreased and replaced with a corresponding concentration of choline chloride (Choi 1987).

The effect of monensin on the stability of mtDNA- and nDNA-encoded transcripts was determined by adding the transcriptional inhibitor actinomycin D to the cultures at a final concentration of 5 μg/mL. After 1 h, either vehicle or monensin was added. Total RNA was isolated at various times over timed periods and processed for northern blot analysis.

Exposure of neurons to glutamate

On day 8 in culture, the cell culture medium was removed and stored. Neuronal cultures were washed once with prewarmed (37°C) Locke solution (134 mM NaCl, 5 mM KCl, 4 mM NaHCO₃, 5 mM HEPES, pH 7.4, 2.3 mM CaCl₂ and 5 mM glucose) and incubated for 15 min. Immediately thereafter, L-glutamate (100 μM) and glycine (10 μM) were added, and the cells were further incubated for 30 min at room temperature (24°C). The cells then were washed

and kept in the old culture medium without glutamate for up to 24 h. Control cultures were treated with vehicle over the same time period as that of glutamate-treated cells. At timed points over an 8-h period, neuronal cultures were washed with DPBS; total RNA was isolated and subjected to northern blot analysis.

Cell viability

Cell viability was determined using a two-color fluorescence assay based on the simultaneous determination of live and dead cells. Two probes were employed that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. Viable cells were quantified after staining of cells with cell-permeant dye calcein AM (2 μ M). Non-viable cells were quantified with cell-impermeant propidium iodide (10 μ g/mL). The ratio of the number of cells that displayed propidium iodide fluorescence (non-viable) to the total number of cells in a field was determined.

RNA analysis

Some 2–10 μ g total RNA was run on a 1.2% formaldehyde agarose gel, stained with ethidium bromide. 28S rRNA and 18S rRNA were imaged and quantified to confirm equal loading of RNA, and the gel was then transferred on to a GeneScreen Plus membrane as described by the manufacturer (Dupont, New England Nuclear, MA, USA). Prehybridization and hybridization were done with Hybridizol reagent (Hybridizol I and II in a ratio of 3 : 2; Serologicals Corporation, Norcross, GA, USA). The blots were prehybridized at 37°C for 16 h and hybridized with ³²P-labeled mtDNA- and nDNA-encoded gene probes at 37°C for 48 h (Chandrasekaran *et al.* 1994). The blots were washed with increasing stringency and the final wash was performed at 65°C with 0.2 \times SSC (1 \times SSC contains 150 mM sodium chloride and 15 mM sodium citrate) and 1% sodium dodecyl sulfate. The blots were exposed to X-ray film (Bio-max MS, Kodak, Rochester, NY, USA) with an intensifying screen for 45 min to 2 days at –70°C. The probes were removed from the blots by placing them in boiling Diethyl Pyrocarbonate (DEPC)-treated water for 10 min. The blots were then rehybridized with a ³²P-labeled control β -actin probe as described above. Finally the blots were hybridized with 12S rRNA probe. The level of RNA hybridized was quantified using an image analysis program. To maintain measured intensities within the linear range, the blots hybridized with different probes were exposed for different periods of time. The level of RNA was quantified from autoradiograms of lower exposure than was used for photography. Ratios of mt-mRNA to β -actin mRNA or 12S rRNA to β -actin mRNA were calculated (Chandrasekaran *et al.* 1994).

Probe preparation and labeling

Mitochondrial DNA probes that simultaneously detect several mt-mRNAs were used (Murdock *et al.* 1999). The probes were created by amplifying nucleotides (nt) 3351–7570 of mtDNA (probe 1) and nt 8861–14 549 of mtDNA (probe 2). Probe 1 hybridized to mtDNA-encoded NADH dehydrogenase (ND) subunit 1 (ND1), ND2, cytochrome oxidase (COX) subunit I (COX I) and COX II mRNAs. Probe 2 hybridized to ND5, ND4 and ND4L, cytochrome *b* (*cyt b*) and COX III mRNAs. cDNA inserts of β -actin cDNA and 12S rRNA clones were used as probes. In the case of nDNA-encoded cytochrome *c* (*cyt c*), COX IV and succinate dehydrogenase (SDH) subunit B (SDH B), probes were prepared by RT-PCR

using gene-specific primers and were confirmed by sequencing. The probes were radiolabeled by the random primer method.

Estimation of half-lives of mt-mRNAs, 12S rRNA and β -actin mRNA

Some 2–10 μ g total RNA from cells treated with either vehicle or monensin in the presence of actinomycin D was subjected to northern blot analysis. The blots were hybridized with mtDNA probe 2, 12S rRNA, β -actin, COX IV and COX VIII probes, and the levels of respective RNA species quantified. Levels of β -actin mRNA are expressed as a percentage of the β -actin mRNA remaining at each experimental time compared with time zero. Levels of mt-mRNA, 12S rRNA, COX IV mRNA and COX VIII mRNA were calculated as the ratio of the respective species to the level of β -actin mRNA. At each experimental time, the RNA ratios are expressed as a percentage of the ratio at time zero. The half-lives were determined from the equation $t_{1/2} = 0.301/\text{slope of the best fit line} (\log_{10} \text{ remaining RNA vs. time})$.

Measurement of ATP and intracellular sodium ($[Na^+]_i$)

Cellular ATP levels were determined luminometrically (Victor³, Shelton, CT, USA); Perkin-Elmer Instruments using an ATP Bioluminescence Assay Kit (Boehringer Ingelheim, Ridgefield, CT, USA) according to the protocol provided. Briefly, after exposing PC12S cells to vehicle, monensin or 2-deoxy-D-glucose, the medium was withdrawn, the cells were exposed to lysis buffer mixed with dilution buffer for 5 min and were then harvested by scraping. Aliquots of cellular extracts were assayed for ATP content using the ATP dependency of the light-emitting luciferase/luciferin reagent. ATP concentration was determined from a standard curve. Results were normalized with respect to cell protein concentration.

The culture medium was replaced with medium containing ²²Na (5 μ Ci or 185 KBq per ml). Measurement of intracellular ²²Na showed that equilibration between added radioactive label and 'cold' sodium in the medium was achieved within 24 h. The cells were then treated for various time periods with either the vehicle or monensin (250 nM). The reaction was terminated by aspiration of the medium, and the cells were quickly washed twice with ice-cold DPBS and digested for 1 h in 0.2 mL 1 M NaOH at room temperature. Cell digests were assayed for ²²Na content by scintillation counting.

Statistical analysis and replication of results

The results presented are representative of at least three to five independent experiments. Where indicated, statistical analysis was carried out using one-way ANOVA followed by Tukey's test for multiple comparisons. Mean \pm SEM values are presented. Differences were considered significant when $p < 0.05$.

Results

Treatment of PC12S cells with monensin reduces cellular ATP levels

Exposure of PC12S cell cultures to monensin (250 nM) caused a sustained 13–60% decrease in total cellular ATP levels, from 15 ± 2 nmol per mg protein at time zero, to

13 ± 1.5, 10 ± 1, 8 ± 0.7 and 5 ± 0.5 nmol per mg protein at 0.5, 1, 3 and 6 h respectively. Exposure of PC12S cells to monensin for 6 h resulted in a 500% increase in intracellular ^{22}Na ($[\text{Na}^+]_i$) from 30 ± 5 to 150 ± 12 pmol per µg protein. Determination of cell viability using calcein AM and propidium iodide showed no decrease in viability (> 95% propidium iodide negative) with exposure to monensin for up to 6 h. Our interpretation of these results is that exposure to monensin in PC12S cells causes an influx of Na^+ , which in turn activates Na/K-ATPase, leading to increased consumption of ATP and decreased cellular ATP levels.

Biphasic changes in expression of mt-mRNAs induced by exposure of PC12S cells to monensin

To examine the effects of increased $[\text{Na}^+]_i$ on mt-mRNA expression, monensin (250 nM) was added to PC12S cell cultures. At different periods of exposure to monensin total cellular RNA was isolated and subjected to northern blotting. Blots of RNA were probed with mtDNA-derived probes (probes 1 and 2), 12S rRNA, nDNA-derived cDNA encoding cyt *c*, COX IV, SDH (Complex II of OXPHOS) subunits A and B (SDH A and B), and β -actin. Levels of β -actin mRNA were determined as a reference to ensure that equivalent amounts of RNA were loaded and transferred into each lane in northern blot analyses (Fig. 1).

Exposure of PC12S cells to monensin increased the mt-mRNA levels during the first hour of treatment (Fig. 1). Continued exposure to monensin caused a significant decrease in mt-mRNAs at 4–6 h (Fig. 1). Levels of

mtDNA-encoded 12S rRNA and nDNA-encoded cyt *c*, COX IV, SDH A, SDH B and β -actin mRNA were unaffected by monensin treatment. Quantification of the mt-mRNA levels showed a biphasic response following exposure to monensin; a significant 1.5-fold increase in mt-mRNAs during the first 30 min of exposure followed by a significant (50%) decrease at 4–6 h after exposure (Figs 1a and b).

Biphasic changes in expression of mt-mRNAs induced by exposure of rat cerebellar granule neurons to glutamate

Although PC12 pheochromocytoma cells represent a cell line commonly used to model neuronal activities, it is possible that the response of mt-mRNA to increased $[\text{Na}^+]_i$ is not the same as in primary cultures of neurons. We therefore tested the effects of the excitatory neurotransmitter glutamate on primary cultures of rat cerebellar granule neurons. Glutamate-induced excitotoxicity in primary neuronal cultures is associated with increases in $[\text{Na}^+]_i$ and intracellular calcium concentration $[\text{Ca}^{2+}]_i$, and decreased cellular ATP levels (Ankarcrona *et al.* 1995). Rat cerebellar neurons were treated with glutamate (100 µM) for 30 min, followed by normal culture conditions. Total cellular RNA was isolated and subjected to northern blot analysis with mtDNA probes at the end of the glutamate exposure and at several times during the 8-h after treatment. An autoradiogram and quantitative data are shown in Fig. 2. Immediately following exposure of neurons to glutamate, levels of mt-mRNA rose significantly (150%). This increase was followed by a significant decrease

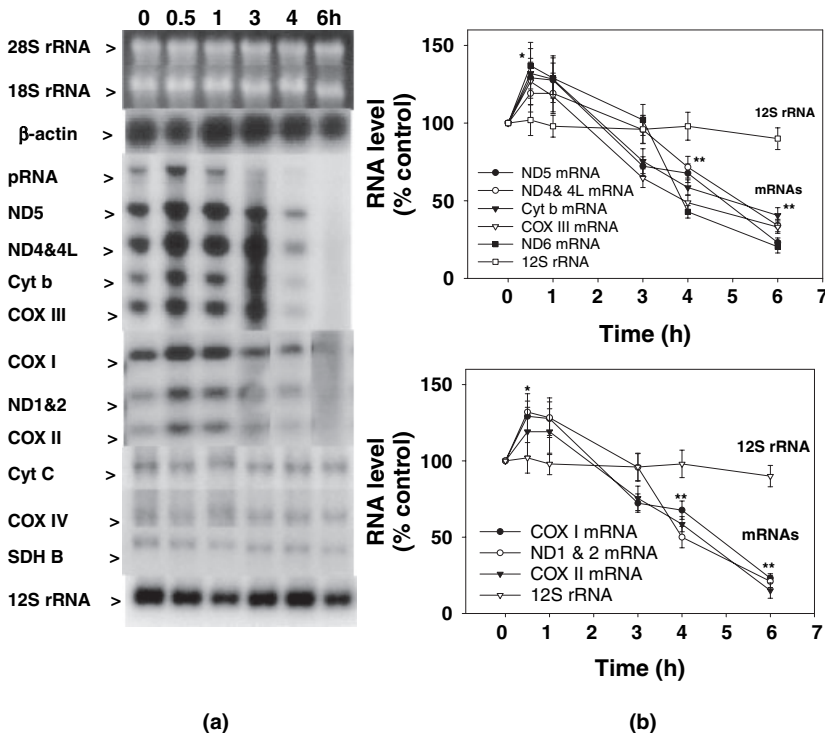


Fig. 1 Effect of monensin on mtDNA- and nDNA-encoded RNA expression in PC12S cells. PC12S cells were exposed to monensin (final concentration 250 nM) for the indicated periods and total RNA was isolated. (a) Northern blot analysis for mtDNA- and nDNA-encoded RNAs. (b) The intensity of the hybridization signal was quantified by image analysis of the autoradiograms. The ratio of individual mtDNA-encoded mRNA and 12S rRNA to β -actin mRNA was calculated at each time point. The percentage RNA change with respect to time zero is shown. Each point represents the mean ± SEM of five separate experiments. No significant changes were observed with mtDNA-encoded 12S rRNA or with nDNA-encoded cyt *c*, COX IV and SDH B mRNAs. pRNA, precursor RNA. * $p < 0.05$, ** $p < 0.01$ versus time zero samples (one-way ANOVA followed by Tukey's test for multiple comparisons).

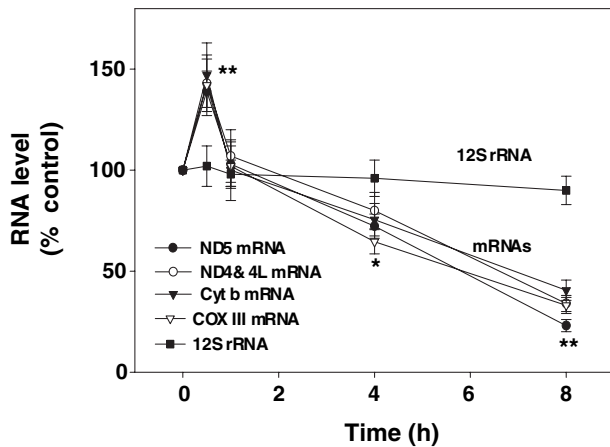
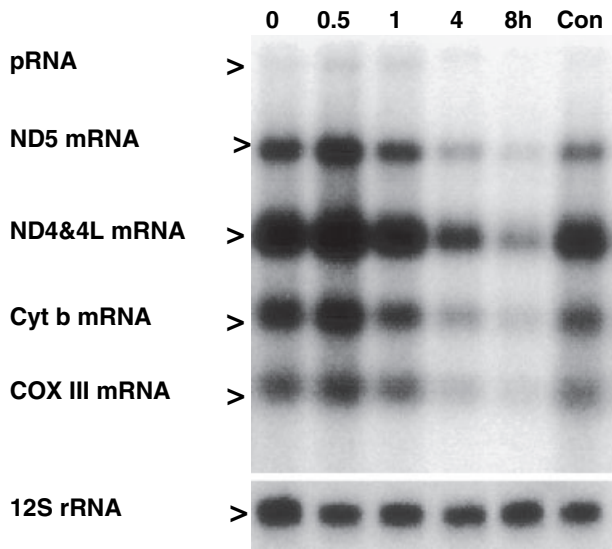


Fig. 2 Effect of glutamate exposure on mtDNA-encoded RNA expression in rat cerebellar neurons. Rat cerebellar granule neurons cultured for 8 days *in vitro* were exposed to glutamate (100 μ M) for 30 min in Locke solution. The cells were then washed and kept in the old culture medium without glutamate for up to 8 h. At indicated times, total RNA was isolated and subjected to northern blot analysis with mtDNA probes. The intensity of the hybridization signal was quantified by image analysis of the autoradiograms. The ratio of individual mt-mRNA and 12S rRNA to β -actin mRNA was calculated at each time point. The percentage RNA change with respect to time zero is shown. Each point represents the mean \pm SEM of three separate experiments. pRNA, precursor RNA. * $p < 0.05$, ** $p < 0.01$ versus time zero (one-way ANOVA followed by Tukey's test for multiple comparisons). Con = 8 h control without glutamate exposure.

at 4–8 h (< 25% compared with level at time zero). These results suggest that there are two mechanisms operating in the regulation of mt-mRNA expression in neuronal cells under conditions of ATP depletion and ionic stress: an early increase in mt-mRNA levels and a decrease at later time periods (> 4 h). We therefore studied the molecular basis of these two mechanisms.

Monensin-induced increase in mt-mRNA levels is abolished by decreasing $[\text{Na}^+]_e$

To understand the relation between the monensin-induced increase in mt-mRNA and changes in $[\text{Na}^+]_i$ or cellular ATP levels, we exposed the PC12S cell cultures to varying concentrations of $[\text{Na}^+]_e$ in the presence of monensin and measured changes in mt-mRNA levels. $[\text{Na}^+]_e$ was decreased from 125 mM to 62.5 mM or to 31.25 mM. Monensin (250 nM) was added to the culture medium and at various times, total cellular RNA was isolated and subjected to northern blot analysis with mtDNA probe. The results shown in Fig. 3 show that when $[\text{Na}^+]_e$ was reduced from 125 mM to either 62.5 or 31.25 mM, the rapid increase and the subsequent decrease in mt-mRNA was eliminated. The effects of monensin on levels of mt-mRNA are therefore sodium dependent.

Addition of ouabain or the calcium ionophore ionomycin does not increase mt-mRNAs

Further experiments were conducted to determine whether the effects of monensin on mt-mRNA levels are due to increased $[\text{Na}^+]_i$ *per se*, or to the reduction in ATP caused by increased sodium cycling. In addition, the possible secondary effect of increased $[\text{Ca}^{2+}]_i$ caused by raised $[\text{Na}^+]_i$ through $\text{Na}^+/\text{Ca}^{2+}$ exchange was investigated (Fasolato *et al.* 1991). To assess the effect of $[\text{Na}^+]_i$, PC12S cells were treated with the Na/K-ATPase inhibitor ouabain (1 mM), which increases $[\text{Na}^+]_i$ without decreasing ATP (Taurin *et al.* 2003). The possible effects of raised $[\text{Ca}^{2+}]_i$ were assessed by the addition of the calcium ionophore ionomycin (3 μ M) (Fasolato *et al.* 1991). Total RNA was isolated and subjected to northern blot analysis with mtDNA probes. The results are shown in Fig. 4.

Addition of ouabain decreased steady-state levels of mt-mRNA. Mitochondrial DNA-encoded 12S rRNA, however, was unaffected by ouabain. Exposure to ouabain for 2 h increased $[\text{Na}^+]_i$ from 30 ± 5 to 75 ± 9 pmol per μ g protein and increased cellular ATP from 15 ± 2 to 23 ± 3 nmol per mg protein. Exposure to ionomycin for up to 2 h caused no significant change in cellular ATP (14.1 ± 2 to 12.2 ± 3 nmol per mg protein) and no significant change in the levels of mt-mRNA. These results indicate that the initial increase in mt-mRNA synthesis in response to monensin is not due to the increase in $[\text{Na}^+]_i$ or $[\text{Ca}^{2+}]_i$ but probably results from decreased cellular ATP levels.

A decrease in cellular ATP does not decrease mt-mRNAs

To determine whether the delayed decrease in mt-mRNA is also due to a decrease in cellular ATP levels, we exposed PC12S cells to 2-deoxy-D-glucose (4.5 g/L) in glucose-free DMEM containing sodium pyruvate (110 mg/L). Cellular ATP levels were determined at several times during the 8 h after treatment. Exposure of PC12S cell cultures to 2-deoxy-D-glucose in glucose-free medium caused a

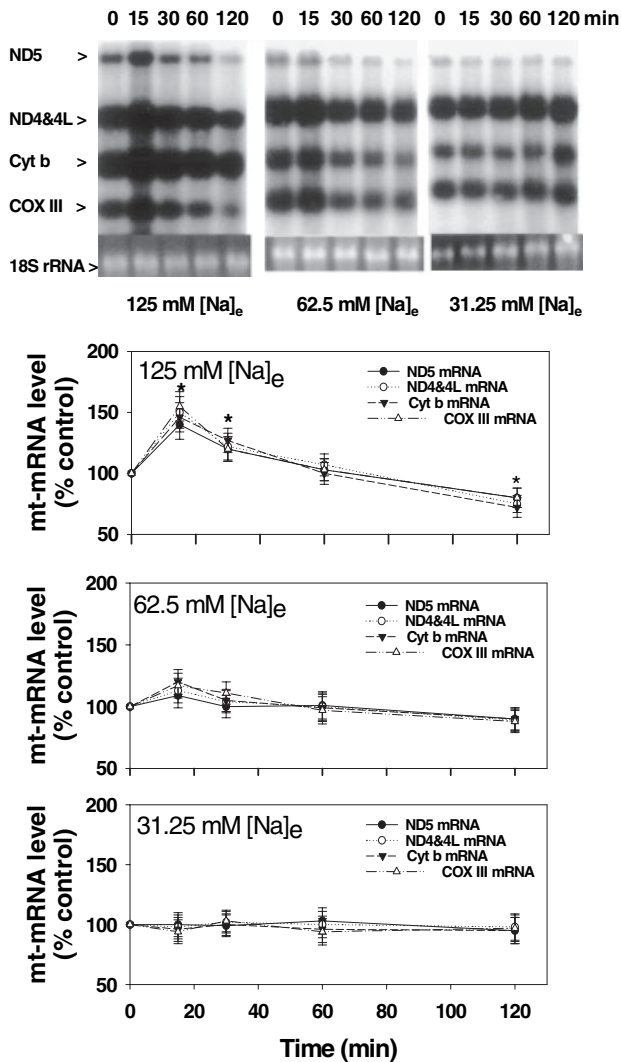


Fig. 3 Effect of changes in extracellular sodium on mtDNA-encoded RNA expression. PC12S cells were exposed to monensin (final concentration 250 nM) in the presence of decreasing $[Na^+]_e$. At indicated times, total RNA was isolated and subjected to northern blot analysis with mtDNA-encoded probes. The intensity of the hybridization signal was quantified by image analysis of the autoradiograms. The ratio of individual mt-mRNA to β -actin mRNA was calculated at each time point. The percentage RNA change with respect to time zero is shown. Note no significant increase was seen at 15 min when the $[Na^+]_e$ was decreased from 125 to 31.2 mM. Each point represents the mean \pm SEM of three separate experiments. * $p < 0.05$ versus time zero (one-way ANOVA followed by Tukey's test for multiple comparisons).

sustained 10–50% decrease in total cellular ATP levels, from 14.2 ± 1.7 nmol per mg protein at time zero, to 13 ± 1 , 10.5 ± 0.9 , 8.1 ± 0.8 and 7.1 ± 0.8 nmol per mg protein at 1, 3, 5 and 8 h respectively (Fig. 5). To examine the effects of decreased cellular ATP levels on mt-mRNA expression, total cellular RNA was isolated from 2-deoxy-

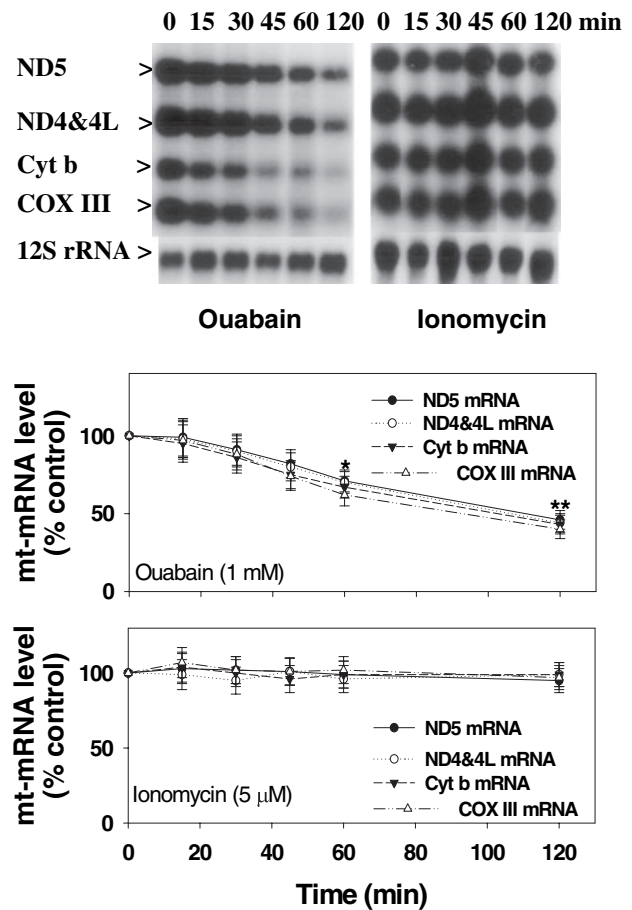


Fig. 4 Effect of ouabain or ionomycin on mtDNA-encoded RNA expression. PC12S cells were exposed to ouabain (final concentration 1 mM) or ionomycin (final concentration 3 μ M) for the indicated time periods. Total RNA was isolated and subjected to northern blot analysis with mtDNA-encoded probes. The intensity of the hybridization signal was quantified by image analysis of the autoradiograms. The ratio of individual mt-mRNA and 12S rRNA to β -actin mRNA was calculated at each time point. The percentage RNA change with respect to time zero is shown. Mt-mRNA levels decreased in ouabain-treated cells, whereas no significant changes were observed with mtDNA-encoded 12S rRNA. Exposure to ionomycin did not alter the levels of mt-mRNA and 12S rRNA during the 2-h period. Each point represents the mean \pm SEM of three separate experiments. * $p < 0.05$, ** $p < 0.01$ versus time zero (one-way ANOVA followed by Tukey's test for multiple comparisons).

D-glucose-treated cells, subjected to northern blotting, and probed with mtDNA-derived probe 2. The autoradiograms of northern blots and the quantification of changes in mt-mRNA levels are shown in Fig. 5. Exposure to 2-deoxy-D-glucose increased the levels of mt-mRNA by 20–32% at 5 and 8 h after treatment and there was no evidence of a decrease in mt-mRNA levels. Thus, the late decrease in mt-mRNA levels in monensin-treated cells appears to be independent of cellular ATP decline.

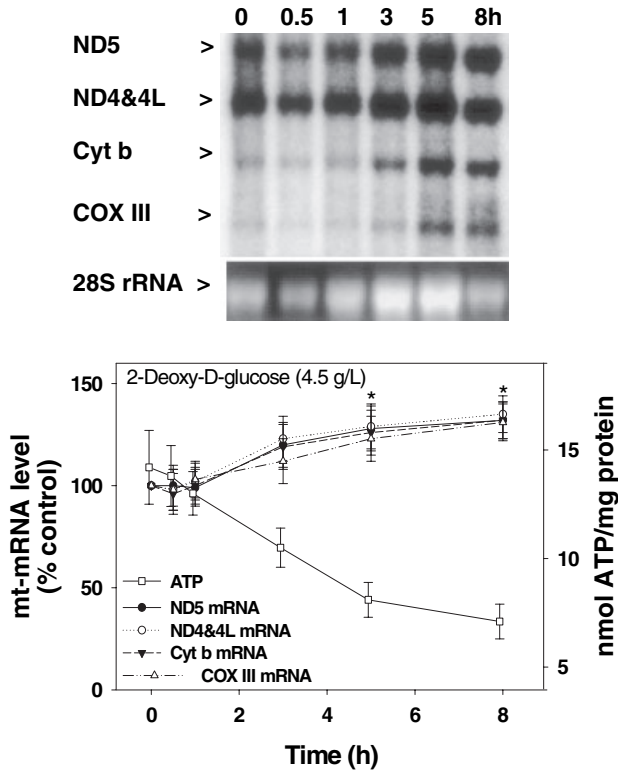


Fig. 5 Effect of 2-deoxy-D-glucose on mtDNA-encoded RNA expression in PC12S cells. PC12S cells were exposed to 2-deoxy-D-glucose (final concentration 4.5 g/L) for the indicated periods, and total RNA was isolated and subjected to northern blot analysis with mtDNA-encoded probes. The intensity of the hybridization signal was quantified by image analysis of the autoradiograms. The ratio of individual mt-mRNA to 18S rRNA was calculated at each time point. The percentage RNA change with respect to time zero is shown. Each point represents the mean \pm SEM of five separate experiments. No significant changes were observed with mt-mRNA at earlier time points (< 3 h), whereas there was a significant increase at later time points (> 5 h). * p < 0.05 versus time zero (one-way ANOVA followed by Tukey's test for multiple comparisons).

Addition of actinomycin D abolishes the monensin-induced initial increase in mt-mRNAs

To test whether the initial increase in mt-mRNAs in monensin-treated cells observed during the first hour is due to increased transcription or decreased mRNA turnover, we determined the effects of adding actinomycin D, an inhibitor of cellular transcription (Chrzanowska-Lightowler *et al.* 1994), on mt-mRNA levels. Actinomycin D (5 μ g/mL) was added to the culture medium and monensin was added 1 h later. Total cellular RNA was isolated at 15, 30, 45, 60 and 120 min, and subjected to northern blot analysis with mtDNA probe 2 (Fig. 6). Throughout this period, cell viability was not compromised and there was no substantial reduction in total RNA yield. The autoradiograms of northern blots probed with mtDNA probe 2 and 12S rRNA, and the quantification of changes in mt-mRNA levels, are shown in

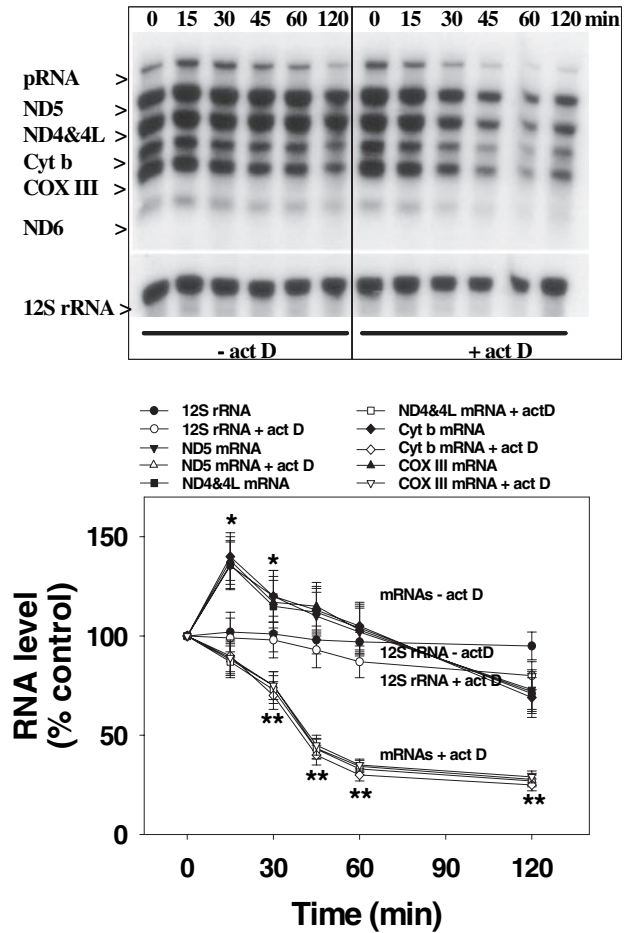


Fig. 6 Effect of termination of transcription with actinomycin D (act D) on monensin-induced increase in mtDNA-encoded RNA levels. PC12S cells were exposed to monensin in the presence or absence of actinomycin D (5 μ g/mL). Total RNA was isolated at indicated times and 2- μ g aliquots were subjected to northern blot analysis. Hybridization was quantified by image analysis of autoradiograms. The ratio of individual mt-mRNA and 12S rRNA to β -actin mRNA was calculated at each time point. The percentage RNA change with respect to time zero is shown. Each point represents the mean \pm SEM of three separate experiments. pRNA, precursor RNA. * p < 0.05, ** p < 0.01 versus time zero (one-way ANOVA followed by Tukey's test for multiple comparisons).

Fig. 6. In the absence of actinomycin D, levels of mt-mRNAs increased as early as 15 min after addition of monensin (Fig. 6). In the presence of actinomycin D, the monensin-induced increase was abolished (Fig. 6). This result suggests that the monensin-induced early increase in mt-mRNA levels is due to increased mtDNA transcription.

Addition of actinomycin D accelerates monensin-induced delayed decrease in mt-mRNAs

To test whether the delayed decrease in mt-mRNA in monensin-treated cells is due to increased degradation or

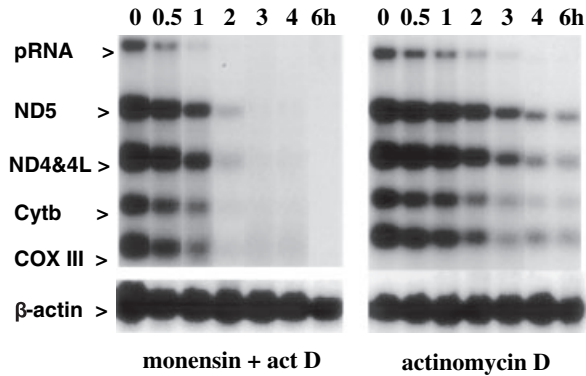


Fig. 7 Effect of actinomycin D on β -actin mRNA and mtDNA-encoded mRNAs in control and monensin-treated PC12S cells. Total RNA was isolated from control and monensin-treated cells at indicated times after termination of transcription by actinomycin D, and 10- μ g aliquots were subjected to northern blot analysis. Hybridization was quantified by image analysis of autoradiograms and normalized to that of β -actin mRNA. Calculated half-lives are shown in Table 1.

Table 1 Estimation of half-lives for β -actin mRNA, 12S rRNA and mtDNA- and nDNA-encoded mRNAs in control and monensin-treated PC12S cells

Transcript	Estimated $t_{1/2}$		Relative $t_{1/2}$ (control/monensin)
	Control	+ Monensin	
Encoded by nDNA			
COX IV	23.4 \pm 5 (3)	20.0 \pm 4 (3)	1.2
COX VIII	24.0 (1)	20.0 (1)	1.2
β -Actin	24.6 \pm 5 (5)	22.0 \pm 5 (5)	1.2
Encoded by mtDNA			
pRNA	3.6 \pm 1 (3)	1.1 \pm 0.2 (3)*	3.3
COX I	3.8 (2)	1 (2)*	3.8
COX II	3.7 (2)	1.1 (2)*	3.4
COX III	3.5 \pm 0.5 (5)	1 \pm 0.1 (5)*	3.5
ND5	3.5 \pm 0.5 (3)	1 \pm 0.2 (3)*	3.5
ND4 and ND4L	3.5 \pm 0.5 (3)	1 \pm 0.2 (3)*	3.5
Cyt <i>b</i>	3.0 \pm 0.25 (3)	1 \pm 0.2 (3)*	3.0
12S rRNA	20 \pm 4 (5)	18 \pm 3 (5)*	1.1

Total RNA was isolated from control and monensin-treated cells at various times after termination of transcription by actinomycin D, and 10- μ g aliquots were subjected to northern blot analysis. Hybridization was quantified by image analysis of autoradiograms and normalized to that of β -actin mRNA. The half-lives were determined from the equation $t_{1/2} = 0.301/\text{slope of the best fit line} (\log_{10} \text{ remaining RNA vs. time})$. Each point represents the mean \pm SEM of three separate experiments. pRNA, precursor RNA. * $p < 0.01$ versus control (one-way ANOVA followed by Tukey's test for multiple comparisons).

decreased synthesis, we determined the half-lives of mt-mRNA, mtDNA-encoded 12S rRNA, nDNA-encoded COX IV, COX VIII and β -actin mRNA in control and

monensin-treated PC12S cells. Actinomycin D (5 μ g/mL) was added to the culture medium and after 1 h monensin was added. Total cellular RNA was isolated at 0.5, 1, 2, 3, 4 and 6 h after adding monensin, and subjected to northern blot analysis. Hybridization results with mtDNA probe 2 and β -actin are shown in Fig. 7. The half-life of other RNAs is shown in Table 1. In monensin-treated cells, half-lives of mtDNA-encoded ND5, ND4, ND4L, cyt *b* and COX III mRNAs were significantly shortened from approximately 3.5 h to 1 h. No significant reductions in half-life were observed with mtDNA-encoded 12S rRNA and nDNA-encoded COX IV, COX VIII and β -actin mRNA in monensin-treated cells. It is also clear that the half-life of mt-mRNA is much shorter (3.5 h) than that of mtDNA-encoded 12S rRNA, nDNA-encoded OXPHOS mRNA and β -actin mRNA (\sim 20 h).

Discussion

This study examined the involvement of transcriptional and post-transcriptional mechanisms in the regulation of mitochondrial gene expression. The data show that exposure of PC12S cells to the sodium ionophore monensin, or exposure of cerebellar granule neurons to the excitatory amino acid glutamate, induces a selective initial increase followed by a delayed decrease in mt-mRNAs. A similar biphasic response in mt-mRNA levels was also observed when human neuroblastoma SH-SY5Y cells were exposed to monensin or primary cortical neuronal cultures were exposed to glutamate. These results suggest the existence of two pathways that regulate the levels of mt-mRNAs in cells.

Investigation into whether mtDNA-encoded structural or non-mitochondrial RNAs are also regulated in response to monensin revealed that the regulation is specific to mt-mRNAs. Exposure of PC12S cells to monensin or exposure of cerebellar neurons to glutamate caused no change in mtDNA-encoded 12S rRNA levels. These RNA subtype-specific effects may reflect differences in the transcriptional regulation of mtDNA-encoded rRNA and mt-mRNA, as well as differences in their half-lives (20 vs. 3.6 h). Quantification of mtDNA by Southern blot analysis showed similar levels in PC12S cells with or without monensin treatment, suggesting that the changes in mt-mRNA are not due to mitochondrial turnover. We measured the levels of three nDNA-encoded OXPHOS mRNAs (cyt *c*, COX IV and SDH B) as well as β -actin mRNA, to determine whether the mt-mRNA changes were specific or represented a general adaptation to the increased energy demand resulting from exposure to monensin. Transcriptional regulators of nuclear-encoded mitochondrial proteins such as nuclear respiratory factors (NRFs) 1 and 2 have been shown to regulate nDNA-encoded cyt *c*, COX IV, SDH B, and the mitochondrial transcription factor Tfam (Virbasius *et al.* 1993; Kelly and Scarpulla 2004). SDH B of OXPHOS complex II was also chosen because this complex is entirely

coded by nDNA, whereas other OXPHOS complexes (I, III, IV and V) consist of subunits encoded by both mtDNA and nDNA (Attardi and Schatz 1988). Our northern blot results showed no significant changes in the levels of nDNA-encoded OXPHOS and non-OXPHOS mRNAs (β -actin) after exposure to monensin. They suggest that the monensin-induced response is specific to mt-mRNAs. Levels of all mt-mRNAs were altered in a similar manner in both monensin- and glutamate-treated cells. This similarity may reflect the transcriptional mechanism by which each strand of mtDNA is transcribed as a single polycistronic message, following which the message is processed to individual mRNAs by post-transcriptional mechanisms (Attardi *et al.* 1990).

Increasing or decreasing energy demand has been shown, respectively, to up-regulate or down-regulate both mtDNA- and nDNA-encoded OXPHOS genes (Hevner and Wong-Riley 1993; Heddi *et al.* 1999; Murdock *et al.* 1999; Wiesner *et al.* 1999; Zhang and Wong-Riley 2000). For example, under conditions of decreased neuronal activity induced by afferent impulse blockade with monocular tetrodotoxin injection in monkeys, mRNA levels of OXPHOS subunit genes encoded by mtDNA and nDNA were decreased in lateral geniculate nucleus and primary visual cortex within days (Hevner and Wong-Riley 1993; Wong-Riley *et al.* 1997). Removal of the blockade restored basal mRNA levels within days (Hevner and Wong-Riley 1993; Wong-Riley *et al.* 1997). However, in these studies the changes in mt-mRNA were disproportionately larger and occurred earlier than changes in mRNA encoded by nDNA, suggesting that changes in mt-mRNA are more tightly regulated by neuronal activity and energy demand (Hevner and Wong-Riley 1993; Wong-Riley *et al.* 1997). On the other hand, the changes in nDNA-encoded OXPHOS mRNA probably represent compensatory mechanisms that operate more slowly. Because we measured changes over a period of 6 h, we interpret the immediate increase in mt-mRNA in response to monensin or glutamate as representing a mechanism that couples increasing energy demand directly with up-regulated mitochondrial gene expression. Consistent with this interpretation are the results that show increased levels of mtDNA- and nDNA-encoded COX subunit mRNA in primary neuronal cultures in response to KCl-induced depolarization (Zhang and Wong-Riley 2000). It would appear that both NRF-2 α and NRF-2 β respond to increased neuronal activity by translocating from the cytoplasm to the nucleus, where they engage in transcriptional activation of target genes such as Tfam (Zhang and Wong-Riley 2000; Yang *et al.* 2004).

Investigations into the molecular component of this rapid coupling suggested that the monensin-induced decrease in cellular ATP level is probably responsible for the stimulation of mitochondrial transcription. *In organello* transcription experiments with isolated mitochondria show that mitochondrial RNA synthesis is regulated in response to changes in intramitochondrial ATP levels (Gaines *et al.* 1987; Enriquez

et al. 1996; DasGupta *et al.* 2001). A low level of intramitochondrial ATP stimulates mtDNA transcription, whereas a high level suppresses mtDNA transcription, possibly by inhibiting mitochondrial RNA polymerase. This sensitivity to ATP levels represents a mechanism by which energy demand could regulate mtDNA transcription (Enriquez *et al.* 1996; DasGupta *et al.* 2001). Moreover, *in organello* studies using [α - 32 P]UTP showed that at low intramitochondrial ATP levels, mRNA species are labeled to a substantial extent, whereas there is minimal labeling of the rRNA species (Gaines *et al.* 1987). This observation may explain our finding of an increase in mt-mRNA levels but not 12S rRNA in monensin-treated cells. The results demonstrating a reduction in mt-mRNA levels associated with increased ATP levels in ouabain-treated cells are also consistent with this mechanism of gene regulation. Thus, the initial increase in mt-mRNA in monensin-treated cells may represent a physiological coupling mechanism that operates at the transcriptional level, allowing mtDNA to generate optimal levels of mRNA in response to energy demands.

Our results demonstrating a rapid positive effect of monensin on transcription of mt-mRNAs and a negative effect of ouabain suggest that the metabolic pathways are uniquely organized in mammalian brain so that dynamic local energy demand can be met rapidly by increased OXPHOS (Rapoport 1970; Kato and Lowry 1973; Sokoloff 1991; Wong-Riley *et al.* 1998). At sites of high energy demand, such as at postsynaptic dendrites and axon terminals where Na/K-ATPase and mitochondria are enriched, increased electrical activity causes an increase in [Na $^{+}$], which in turn stimulates Na/K-ATPase to consume ATP. Reduced ATP and increased ADP and AMP level can be sensed by mitochondria to stimulate the transcription and translation of mtDNA-encoded subunits. These changes provide a framework for the import and integration of nuclear-encoded subunits, which are entirely synthesized in cell bodies. Dynamic local energy demand can be met when mtDNA- and nDNA-encoded subunits are assembled to form functional OXPHOS complexes.

The decrease in mt-mRNA at 4–6 h of exposure to monensin suggests the presence of a slower-acting mechanism for inhibiting mitochondrial gene expression, which is independent of the cell energy status and overrides the normal rapid regulation by energy demand. The independence of the decrease in mt-mRNA levels with cellular ATP decline was substantiated by the observation that exposure to 2-deoxy-D-glucose decreased cellular ATP levels with no evidence of any mt-mRNA decrease. Decreases in mt-mRNA levels under pathological conditions have been observed. For example, the level of mtDNA-encoded COX I mRNA decreases within hours in CA1 neurons of gerbils after transient forebrain ischemia (Abe *et al.* 1993). This decrease occurs in the absence of a decrease in mtDNA, suggesting impaired transcription and/or turnover of mt-mRNA (Abe *et al.* 1993).

In this model, the decrease in COX I mRNA occurs when energy demand is high on these cells to restore ionic gradients to resting levels and to maintain neuronal activity (Arai *et al.* 1986; Abe *et al.* 1993). A disproportionate decrease in mtDNA-encoded COX subunit mRNA in the absence of a change in mtDNA-encoded 12S rRNA has also been noted in brains of patients with Alzheimer's disease (Chandrasekaran *et al.* 1994, 1998; Hatanpaa *et al.* 1996).

Our actinomycin D experiments suggest that a post-transcriptional mechanism is responsible for the observed decrease in mt-mRNA in monensin-treated cells. Half-lives of mt-mRNA, 12S rRNA, COX IV, COX VIII and β -actin mRNA in the presence and absence of monensin were calculated after inhibition of *de novo* mitochondrial and nuclear transcription by actinomycin D. The estimated half-life of mt-mRNAs in control cells was ~ 3.6 h, whereas that of mtDNA-encoded 12S rRNA and of nDNA-encoded COX IV, COX VIII and β -actin mRNA was greater than 20 h. The half-lives of mt-mRNAs in PC12S cells are similar to those reported in other cell culture systems (Gelfand and Attardi 1981; Chrzanowska-Lightowlers *et al.* 1994). In monensin-treated cells, the estimated half-life of mt-mRNA decreased from 3.6 to ~ 1 h, representing a 3.5-fold decrease in the stability of mt-mRNA in monensin-treated cells. This post-transcriptional mechanism involving Rnase(s) probably accounts for the accelerated degradation of mt-mRNA.

Although we have not identified a specific Rnase responsible for the accelerated degradation of mt-mRNA in PC12S cells, an Rnase-L has been reported to degrade a number of mitochondrial mRNAs in human H9 cells in response to interferon- α treatment (Le Roy *et al.* 2001). This Rnase-L pathway is activated in models of ischemia-reperfusion injury (Paschen *et al.* 1999). A comparison of the half-life of mt-mRNA in monensin-treated mouse embryo fibroblasts showed greater stability in Rnase-L $^{-/-}$ cells compared with Rnase-L $^{+/+}$ cells (Chandrasekaran *et al.* 2004). Thus, activation of Rnase-L may be responsible for the accelerated degradation of mt-mRNA in monensin-treated cells.

In summary, our results suggest a physiological transcriptional mechanism of regulation of mitochondrial gene expression by energy demand and a pathological post-transcriptional regulation that is independent of energy status of the cell. The post-transcriptional mechanism is likely to be pathological because it overrides the normal regulation by energy demand, causes accelerated degradation of transcripts and undermines the actual energy demand of the cell.

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References

- Abe K., Kawagoe J. and Kogure K. (1993) Early disturbance of a mitochondrial DNA expression in gerbil hippocampus after transient forebrain ischemia. *Neurosci. Lett.* **153**, 173–176.
- Ankarcrona M., Dypbukt J. M., Bonfoco E., Zhivotovsky B., Orrenius S., Lipton S. A. and Nicotera P. (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15**, 961–973.
- Arai H., Passonneau J. V. and Lust W. D. (1986) Energy metabolism in delayed neuronal death of CA1 neurons of the hippocampus following transient ischemia in the gerbil. *Metab. Brain Dis.* **1**, 263–278.
- Attardi G. and Schatz G. (1988) Biogenesis of mitochondria. *Annu. Rev. Cell Biol.* **4**, 289–333.
- Attardi G., Chomyn A., King M. P., Kruse B., Polosa P. L. and Murdter N. N. (1990) Regulation of mitochondrial gene expression in mammalian cells. *Biochem. Soc. Trans.* **18**, 509–513.
- Chandrasekaran K., Giordano T., Brady D. R., Stoll J., Martin L. J. and Rapoport S. I. (1994) Impairment in mitochondrial cytochrome oxidase gene expression in Alzheimer disease. *Brain Res. Mol. Brain Res.* **24**, 336–340.
- Chandrasekaran K., Hatanpaa K., Brady D. R., Stoll J. and Rapoport S. I. (1998) Downregulation of oxidative phosphorylation in Alzheimer disease: loss of cytochrome oxidase subunit mRNA in the hippocampus and entorhinal cortex. *Brain Res.* **796**, 13–19.
- Chandrasekaran K., Mehrabian Z., Li X. L. and Hassel B. (2004) RNase-L regulates the stability of mitochondrial DNA-encoded mRNAs in mouse embryo fibroblasts. *Biochem. Biophys. Res. Commun.* **325**, 18–23.
- Choi D. W. (1987) Ionic dependence of glutamate neurotoxicity. *J. Neurosci.* **7**, 369–379.
- Chrzanowska-Lightowlers Z. M., Preiss T. and Lightowlers R. N. (1994) Inhibition of mitochondrial protein synthesis promotes increased stability of nuclear-encoded respiratory gene transcripts. *J. Biol. Chem.* **269**, 27 322–27 328.
- DasGupta S. F., Rapoport S. I., Gerschenson M., Murphy E., Fiskum G., Russell S. J. and Chandrasekaran K. (2001) ATP synthesis is coupled to rat liver mitochondrial RNA synthesis. *Mol. Cell. Biochem.* **221**, 3–10.
- Enriquez J. A., Fernandez-Silva P., Perez-Martos A., Lopez-Perez M. J. and Montoya J. (1996) The synthesis of mRNA in isolated mitochondria can be maintained for several hours and is inhibited by high levels of ATP. *Eur. J. Biochem.* **237**, 601–610.
- Erecinska M. and Silver I. A. (1989) ATP and brain function. *J. Cereb. Blood Flow Metab.* **9**, 2–19.
- Fasolato C., Zottini M., Clementi E., Zacchetti D., Meldolesi J. and Pozzan T. (1991) Intracellular Ca^{2+} pools in PC12 cells. Three intracellular pools are distinguished by their turnover and mechanisms of Ca^{2+} accumulation, storage, and release. *J. Biol. Chem.* **266**, 20 159–20 167.
- Fukuyama R., Chandrasekaran K. and Rapoport S. I. (1993) Nerve growth factor-induced neuronal differentiation is accompanied by differential induction and localization of the amyloid precursor protein (APP) in PC12 cells and variant PC12S cells. *Brain Res. Mol. Brain Res.* **17**, 17–22.
- Gaines G. and Attardi G. (1984) Highly efficient RNA-synthesizing system that uses isolated human mitochondria: new initiation events and *in vivo*-like processing patterns. *Mol. Cell. Biol.* **4**, 1605–1617.
- Gaines G., Rossi C. and Attardi G. (1987) Markedly different ATP requirements for rRNA synthesis and mtDNA light strand transcription versus mRNA synthesis in isolated human mitochondria. *J. Biol. Chem.* **262**, 1907–1915.

- Gelfand R. and Attardi G. (1981) Synthesis and turnover of mitochondrial ribonucleic acid in HeLa cells: the mature ribosomal and messenger ribonucleic acid species are metabolically unstable. *Mol. Cell. Biol.* **1**, 497–511.
- Hatanpaa K., Brady D. R., Stoll J., Rapoport S. I. and Chandrasekaran K. (1996) Neuronal activity and early neurofibrillary tangles in Alzheimer's disease. *Ann. Neurol.* **40**, 411–420.
- Heddi A., Stepien G., Benke P. J. and Wallace D. C. (1999) Coordinate induction of energy gene expression in tissues of mitochondrial disease patients. *J. Biol. Chem.* **274**, 22 968–22 976.
- Hevner R. F. and Wong-Riley M. T. (1993) Mitochondrial and nuclear gene expression for cytochrome oxidase subunits are disproportionately regulated by functional activity in neurons. *J. Neurosci.* **13**, 1805–1819.
- Hevner R. F., Liu S. and Wong-Riley M. T. (1995) A metabolic map of cytochrome oxidase in the rat brain: histochemical, densitometric and biochemical studies. *Neuroscience* **65**, 313–342.
- Kagawa Y. and Ohta S. (1990) Regulation of mitochondrial ATP synthesis in mammalian cells by transcriptional control. *Int. J. Biochem.* **22**, 219–229.
- Kato T. and Lowry O. H. (1973) Enzymes of energy-converting systems in individual mammalian nerve cell bodies. *J. Neurochem.* **20**, 151–163.
- Kelly D. P. and Scarpulla R. C. (2004) Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev.* **18**, 357–368.
- Le Roy F., Bisbal C., Silhol M., Martinand C., Lebleu B. and Salehzada T. (2001) The 2–5A/RNase L/RNase L Inhibitor (RLI) pathway regulates mitochondrial mRNAs stability in interferon alpha-treated H9 cells. *J. Biol. Chem.* **276**, 48 473–48 482.
- Liu L. I., Rapoport S. I. and Chandrasekaran K. (1999) Regulation of mitochondrial gene expression in differentiated PC12 cells. *Ann. N. Y. Acad. Sci.* **893**, 341–344.
- Murdock D. G., Boone B. E., Esposito L. A. and Wallace D. C. (1999) Up-regulation of nuclear and mitochondrial genes in the skeletal muscle of mice lacking the heart/muscle isoform of the adenine nucleotide translocator. *J. Biol. Chem.* **274**, 14 429–14 433.
- Paschen W., Althausen S. and Douthel J. (1999) Ischemia-induced changes in 2'-5'-oligoadenylate synthetase mRNA levels in rat brain: comparison with changes produced by perturbations of endoplasmic reticulum calcium homeostasis in neuronal cell cultures. *Neurosci. Lett.* **263**, 109–112.
- Pressman B. C. and Fahim M. (1982) Pharmacology and toxicology of the monovalent carboxylic ionophores. *Annu. Rev. Pharmacol. Toxicol.* **22**, 465–490.
- Purdon A. D., Rosenberger T. A., Shetty H. U. and Rapoport S. I. (2002) Energy consumption by phospholipid metabolism in mammalian brain. *Neurochem. Res.* **27**, 1641–1647.
- Rapoport S. I. (1970) The sodium–potassium exchange pump: relation of metabolism to electrical properties of the cell. I. Theory. *Biophys. J.* **10**, 246–259.
- Schousboe A., Meier E., Drejer J. and Hertz L. (1989) Preparation of primary cultures of mouse (rat) cerebellar granule cells, in *A Dissection and Tissue Culture Manual of the Nervous System* (Shahar A., de Vellis J., Vernadakis A. and Haber B., eds), pp. 203–206. Alan R. Liss, Inc., New York.
- Sokoloff L. (1991) Measurement of local cerebral glucose utilization and its relation to local functional activity in the brain. *Adv. Exp. Med. Biol.* **291**, 21–42.
- Taurin S., Hamet P. and Orlov S. N. (2003) Na/K pump and intracellular monovalent cations: novel mechanism of excitation–transcription coupling involved in inhibition of apoptosis. *Mol. Biol. (Mosk.)* **37**, 371–381.
- Virbasius C. A., Virbasius J. V. and Scarpulla R. C. (1993) NRF-1, an activator involved in nuclear–mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. *Genes Dev.* **7**, 2431–2445.
- Wiesner R. J., Hornung T. V., Garman J. D., Clayton D. A., O'Gorman E. and Wallimann T. (1999) Stimulation of mitochondrial gene expression and proliferation of mitochondria following impairment of cellular energy transfer by inhibition of the phosphocreatine circuit in rat hearts. *J. Bioenerg. Biomembr.* **31**, 559–567.
- Wong-Riley M. T. (1989) Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends Neurosci.* **12**, 94–101.
- Wong-Riley M. T., Mullen M. A., Huang Z. and Guyer C. (1997) Brain cytochrome oxidase subunit complementary DNAs: isolation, subcloning, sequencing, light and electron microscopic *in situ* hybridization of transcripts, and regulation by neuronal activity. *Neuroscience* **76**, 1035–1055.
- Wong-Riley M., Anderson B., Liebl W. and Huang Z. (1998) Neurochemical organization of the macaque striate cortex: correlation of cytochrome oxidase with Na⁺K⁺ATPase, NADPH-diaphorase, nitric oxide synthase, and N-methyl-D-aspartate receptor subunit 1. *Neuroscience* **83**, 1025–1045.
- Yang S. J., Liang H. L., Ning G. and Wong-Riley M. T. (2004) Ultrastructural study of depolarization-induced translocation of NRF-2 transcription factor in cultured rat visual cortical neurons. *Eur. J. Neurosci.* **19**, 1153–1162.
- Zhang C. and Wong-Riley M. T. (2000) Synthesis and degradation of cytochrome oxidase subunit mRNAs in neurons: differential bigenomic regulation by neuronal activity. *J. Neurosci. Res.* **60**, 338–344.