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Hypoxic Coordinate Regulation of Mitochondrial Enzymes in Mammalian Cells

Abstract. *The effect of hypoxic exposure on various mitochondrial enzymes and on cell mitochondrial genomic content was studied in two types of mammalian cells. Hypoxia depressed the activity of six enzymes to the same degree. The kinetics of depression and of recovery during reexposure to normoxia were statistically similar for three marker enzymes. Despite the global and symmetrical decrease in enzyme activities, mitochondrial DNA remained constant. This suggests either symmetrical loss of mitochondrial enzymes from all mitochondria or complete loss of enzymes from a subpopulation of mitochondria with retention of an intact mitochondrial genome.*

It is generally accepted that there are two major mechanisms for the regulation of overall mitochondrial composition. One of these regulatory mechanisms determines the number of mitochondria per cell. This function is largely related to the rate of mitochondrial replication, which must involve synthesis of mitochondrial DNA (mtDNA) (*1*). Since the various cell types presumably achieve a steady state with respect to mitochondrial number, the rate of mitochondrial degradation must be regulated as well. The second of the major mechanisms regulates individual proteins making up mitochondria. This is accomplished largely through nucleus-directed protein synthesis on cytoplasmic ribosomes, but there is an independent mitochondrial transcription-translation system that regulates the biosynthesis of special mitochondrial components (*2*). Because various mitochondrial components have variable turnover times (*3*), variable degrees of nuclear and mitochondrial control (*4*), and variable specific genomic expression (*5*), individual proteins appear to be individually regulated.

We have obtained evidence that there is another form of regulation. During incubation under hypoxic conditions, various mitochondrial enzymes show a generalized decrease in activity. The magnitudes of these decreases are statistically similar for all enzymes, as are the changes with time in enzyme activity for three index enzymes. This suggests a coordinate regulation of mitochondrial function. Measurements of mtDNA content show that there is no significant change during hypoxic incubation. Hence, coordinate depression of enzyme levels is not simply the result of a reduction in mitochondrial number produced by alterations in the rate of mitochondrial replication or degradation.

The decreases in mitochondrial enzyme activity with an unchanged mtDNA content must mean that there is a general inhibitor of mitochondrial enzyme activity, or that all mitochondria symmetrically and reversibly lose a substantial segment of mitochondrial enzyme content, or that a subpopulation of mitochondria can reversibly revert to an elementary form that lacks mitochondrial enzymes but contains mtDNA.

Two different cell types were studied. Mouse lung macrophages, obtained by lung mincing (*6*) were cultured in a system in which the cells do not replicate. Rat skeletal muscle L8 cells (*7*) in early passage were cultured in a system in which replication does occur. The results were essentially the same for both cell types, indicating that cell replication is not a major factor in coordinate regulation. Each cell type was cultured under normoxic (PO_2, ~ 120 torr; PCO_2, ~ 40 torr) or hypoxic (PO_2, 15 to 25 torr; PCO_2 ~ 40 torr) conditions.

In the first series of studies, enzyme activities of six separate enzymes were measured after 96 hours of incubation. Four enzymes located on the mitochondrial matrix were studied. Three of these—citrate synthetase, nicotinamide-adenine dinucleotide (NAD^+)-dependent isocitrate dehydrogenase, and malate dehydrogenase—participate in the tricarboxylic acid cycle, and glutamate dehydrogenase functions as a link between carbohydrate and amino acid metabolism. Also studied were succinate dehydrogenase, an inner mitochondrial component of the Krebs cycle, and cytochrome aa_3, a component of the inner membrane mitochondrial electron transport chain.

We measured enzyme activities by

Table 1. A comparison of mitochondrial enzyme activities under normoxic (PO_2, 120 torr) and hypoxic (PO_2, 15 to 25 torr) conditions after 96 hours of incubation. All enzyme activities under hypoxic conditions were significantly less ($P < 0.05$ except where noted) than under normoxic conditions ($N = 6$ for all studies except where noted). Analysis of variance revealed that in each cell type the degree of depression of mitochondrial activity for each enzyme was statistically the same with the exception of cytochrome aa_3 in L8 rat skeletal muscle cells. Succinate dehydrogenase activities in mouse lung macrophages were too low to be measured. Values are expressed as the mean ± standard deviation (S.D.).

Enzyme	Enzyme activity (μmole/min-mg)		Percent change
	Normoxia	Hypoxia	
L8 rat skeletal muscle cells			
Citrate synthetase	0.150 ± 0.021	0.105 ± 0.012	29.8 ± 6.9
NAD-isocitrate dehydrogenase	0.014 ± 0.002	0.009 ± 0.002	36.0 ± 13.0
Mitochondrial malate dehydrogenase	3.43 ± 0.41	2.70 ± 0.41	22.7 ± 8.0
Succinate dehydrogenase	0.036 ± 0.006	0.027 ± 0.006	26.3 ± 8.9
Cytochrome aa_3	0.038 ± 0.015	0.021 ± 0.008	42.8 ± 9.4
Glutamate dehydrogenase	0.019 ± 0.004	0.014 ± 0.002	28.2 ± 5.4
Mouse lung macrophages			
Citrate synthetase	0.219 ± 0.047	0.111 ± 0.050	49.4 ± 24.5
NAD-isocitrate dehydrogenase	0.012 ± 0.004	0.006 ± 0.002	49.2 ± 12.7
Mitochondrial malate dehydrogenase*	0.897 ± 0.211	0.580 ± 0.104	39.7 ± 21.1
Cytochrome aa_3	0.198 ± 0.060	0.108 ± 0.045	45.4 ± 15.0
Glutamate dehydrogenase	0.175 ± 0.032	0.102 ± 0.038	48.0 ± 16.5

*For malate dehydrogenase in mouse lung macrophages, $N = 3$, $P < 0.1$.

standard methods (*8*), using cofactor and substrate excess at 37°C, *p*H 7.4, and ionic strength 220 mosmole. Under these conditions, changes in measured activity of an enzyme are paralleled by changes in enzyme content (Table 1). At 96 hours there was a generalized and equivalent reduction of measured enzyme activities. The degree of reduction with one exception (cytochrome aa_3 in L8 cells) was the same as that indicated by an analysis of variance.

These studies establish the coordinate regulation of enzyme activity. To determine the degree of coordinate regulation, studies of the kinetics of coordinate regulation were undertaken. Mouse lung macrophages were incubated under hypoxic conditions for 4 days and then under normoxic conditions for 4 days. The activities of three marker enzymes (citrate synthetase, glutamate dehydrogenase, and cytochrome aa_3) were measured at 48-hour intervals (Fig. 1).

During hypoxic incubation, the decrease in enzyme activity with time was the same for all three marker enzymes. During normoxia the process is reversible. Not only does enzyme activity return to baseline values, but there is an overshoot to approximately 140 percent of baseline values. These results indicate that coordinate regulation by hypoxia occurs not only in degree but also in time.

A relatively simple mechanism for producing these results would involve a decrease in mitochondrial number per cell evoked by O_2 depletion. To explore this possibility, we measured mtDNA in mouse lung macrophages under normoxic and hypoxic conditions using the method of Tapper and Clayton (*9*). A known quantity of a smaller circular plasmid DNA (100 ng of pHRB) was added to each plate of cells prior to extraction of mtDNA by the method of Hirt (*10*). Plasmid pHRB consists of pBR322 with a fragment of mouse mtDNA inserted into the Eco RI and Hind III sites. Closed circular DNA (mitochondrial and pHRB) was separated from nuclear DNA and extracted by the standard phenol-chloroform method (*9*). The mitochondrial DNA and pHRB DNA were then subjected to restriction endonuclease digestion. The enzyme Bgl II was used because each form of DNA exhibits only one such restriction site. The linear forms were then end-labeled with [α-^{32}P]deoxyadenosine triphosphate at 10 μM and unlabeled deoxyguanosine triphosphate at 100 μM. The mixture was subjected to electrophoresis on a 1 percent agarose gel in tris-borate-EDTA buffer for 1500 volt hours. This provided adequate separation of the two DNA species. The bands were then cut from the gel and assayed directly in a scintillation counter. The amount of radioactivity in the mtDNA band relative to the amount in the pHRB band provided a measurement of the content of mtDNA per cell (Table 2). The assay is linear over a range of 10 to 500 ng.

Table 2. Number of mtDNA molecules per cell in nonreplicating lung macrophages. The number of cells was calculated from the total plate DNA content. A two sample *t*-test indicated no significant differences in mtDNA counts during normoxia (PO_2, 120 torr) and hypoxia (PO_2, 25 torr). The activities of two marker enzymes measured simultaneously decreased by 40 to 50 percent during hypoxic incubation.

Study	Number of mtDNA molecules per cell	
	Normoxia	Hypoxia
1	1872	1600
2	1233	950
3	1808	2431
4	2026	1993
5	1804	1771
6	1163	1050
7	2427	1994
Mean ± S.D.	1761 ± 440	1684 ± 533

Despite a decrease of 40 to 50 percent in the measured activities of two marker enzymes (data not shown), there was no significant difference in the cell content of mtDNA under normoxic and hypoxic conditions. It is therefore not possible to attribute the striking coordinate regulation of mitochondrial enzyme activity to a decrease in mitochondrial number per cell.

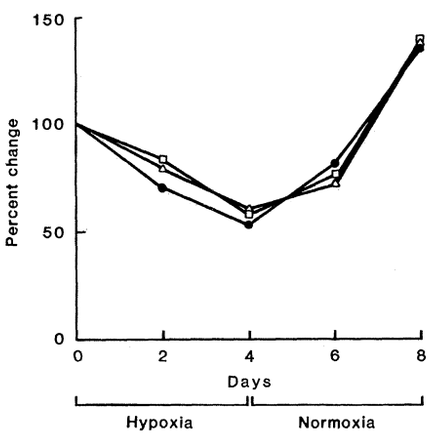

Fig. 1. The kinetics of coordinate regulation of mitochondrial enzymes in mouse lung macrophages. All values were normalized to 100 percent activity per total cell DNA under control conditions. Measurements were performed at 48-hour intervals under hypoxic conditions on days 0 to 4 and under normoxic conditions on days 4 to 8. During the normoxic phase, not only do the enzyme activities return to baseline, but at day 8 there is an overshoot to about 140 percent of the baseline value. An analysis of variance indicates that the rate of change of enzyme activity with time remains the same, including the overshoot phase. (□) Cytochrome oxidase; (△) glutamate dehydrogenase; (●) citrate synthetase.

Our studies show a generalized and simultaneous decrease in the activities of six mitochondrial enzymes during hypoxic incubation. The six enzymes are sufficiently representative so that probably all mitochondrial enzymes are affected in the same way, although it is theoretically possible that some mitochondrial enzymes might not be similarly regulated. Not only do all of the enzymes decrease to the same degree, but the kinetics of the decrease in enzyme activities and their increase during subsequent normoxic reincubation are essentially identical.

We considered the possibility that the decreases in measured activity might have resulted from nonspecific injury or early cell death related to hypoxia. This appears most unlikely. There was no increase in trypan blue uptake by the hypoxic L8 cells. The L8 cells continued to replicate and synthesized cell proteins at a rate equal to that of normoxic cells. Both the L8 cells and the lung macrophages increased the rate of biosynthesis of glycolytic enzymes during hypoxic exposure (*11*). Oxygen consumption was maintained, and the changes were reversible on reoxygenation of the cells. Although none of these is an absolute criterion, it appears that nonspecific injury or early cell death is not a reasonable explanation for the decreases in enzyme activity.

A second possibility is that only measured activity of the enzymes changed and that the content of the various enzymes remained constant. This is theoretically possible, and it is conceivable that there could be a low molecular weight regulator which universally, uniformly, and specifically depresses mitochondrial enzyme activity. Even though it seems unlikely, and preliminary data indicate that the decreases in activity of cytochrome aa_3 are paralleled by decreases in true enzyme content, such a regulator is a formal possibility.

The possibility that a reduction in mitochondrial number per cell is the result either of a decreased rate of biogenesis or an increased rate of degradation of mitochondria is ruled out by the failure of cell mtDNA content to decrease despite the decrease in enzyme activity.

The remaining possibilities are that each mitochondrion shares a proportionate decrease in the contents of its enzymes or that a subpopulation of mito-

chondria reverts to a more elementary state with hypoxic exposure. Examples of the former process have been described in fetal or postnatal mitochondria. For example, there are numerous reports of a perinatal increase in the specific activities of many Krebs cycle and electron chain components (*12*). These changes are usually attributed to mitochondrial proliferation, with increases in inner membrane and enzyme synthesis (*13*).

The possibility of promitochondria is an old suggestion that arose from studies on yeast, which reversibly lose well-developed mitochondria and cytochromes aa_3, b, c, and c_1 (*14*) during anaerobic cultivation. Heyman-Blanchet *et al.* (*15*) reported the isolation of mitochondria-like particles from anaerobic yeast. Wallace and Linnane (*16*) reported that reexposure to O_2 in yeast was associated with numerous electron-transparent vesicles that finally evolved into mitochondria. This finding was supported by some studies and refuted by others.

Even in yeast the possibility of a promitochondrion is not in the main stream of mitochondrial studies. It has not to our knowledge been at all considered in mammalian systems.

Our data do not permit the resolution of the mechanisms of the changes that have been observed, but it is clear that the present findings dictate some modification of current views concerning the basic regulation of mitochondrial composition. The results are inconsistent with individual regulation of mitochondrial proteins and are inconsistent with regulation of mitochondrial number by a simple balance between rates of mitochondrial replication and degradation.

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Intracellular Study of Human Epileptic Cortex: In vitro Maintenance of Epileptiform Activity?

Abstract. *Intracellular recordings were obtained in the in vitro slice preparation from neurons of lateral and mesial temporal cortex removed from human epileptics suffering from intractable temporal lobe seizures. Spontaneous rhythmic synaptic events, which were capable of triggering action potential discharge, were observed in many neurons, particularly in mesial tissue slices. Such activity may reflect the epileptogenic capacity of this human cortex.*

The difficulties involved in investigating basic mechanisms underlying epileptic activity include the ethical and technical problems of obtaining intracellular electrophysiological data from human epileptic brain. Although intracellular data can be collected from animal models (*1*), the questionable relevance of any one model to human epilepsy makes animal results difficult to interpret. Extracellular recordings have been obtained from the human epileptic brain (*2*), but these data cannot be used to reveal underlying mechanisms of abnormal discharge. Development of the in vitro slice preparation (*3*) has made it possible to use intracellular techniques to study human cortical tissue excised

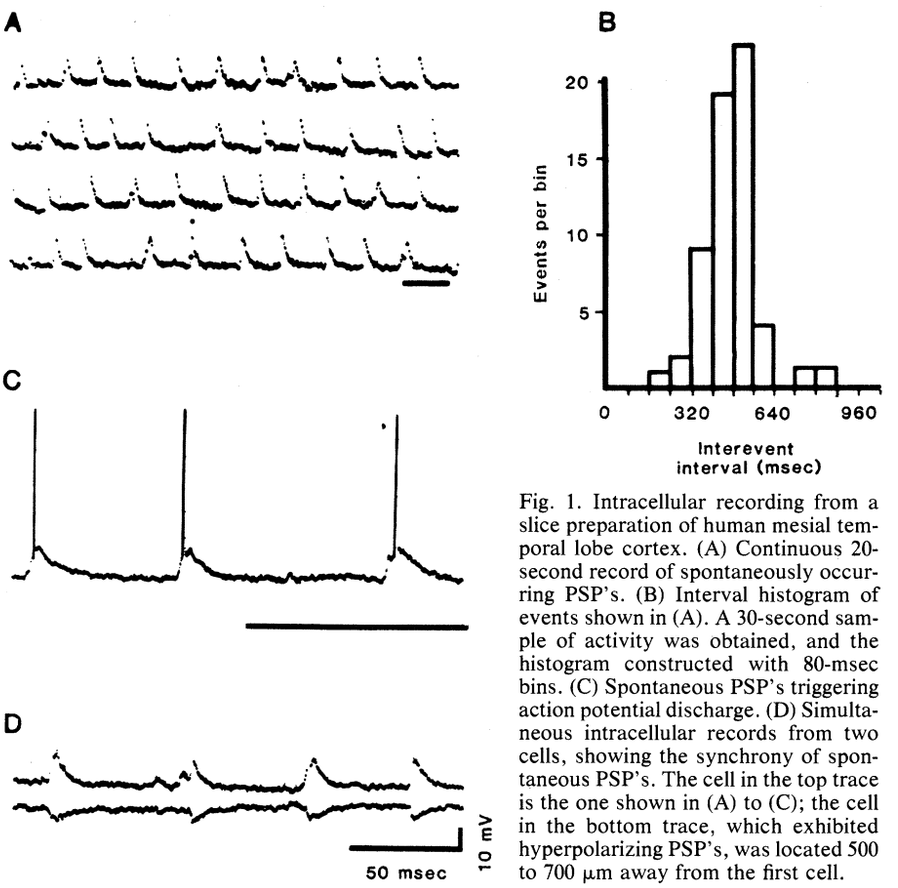

Fig. 1. Intracellular recording from a slice preparation of human mesial temporal lobe cortex. (A) Continuous 20-second record of spontaneously occurring PSP's. (B) Interval histogram of events shown in (A). A 30-second sample of activity was obtained, and the histogram constructed with 80-msec bins. (C) Spontaneous PSP's triggering action potential discharge. (D) Simultaneous intracellular records from two cells, showing the synchrony of spontaneous PSP's. The cell in the top trace is the one shown in (A) to (C); the cell in the bottom trace, which exhibited hyperpolarizing PSP's, was located 500 to 700 μm away from the first cell.