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Mitochondrial functions during experimentally induced cardiac hypertrophy

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Abstract. When the hearts of albino rats are subjected to pressure-induced stress through constriction of ascending aorta, changes in the mitochondrial functions are observed as early as 24 h after the imposition of the stress. These include the abolition of oxidative phosphorylation, decrease in the energy dependent [^{45}Ca]-uptake and decrease in the rate of energized swelling. A large influx of calcium ions and an increase in the fluidity of mitochondrial membranes also occur in this period. At later stages of hypertrophy (17, 28, 40%), these mitochondrial functions gradually return to normal levels.

Keywords. Cardiac hypertrophy; mitochondria; oxidative phosphorylation; respiratory control index; Arrhenius plot; energized swelling.

Introduction

Mitochondria of various tissues respond differentially to different types of stress with regard to the bioenergetic functions such as oxidative phosphorylation, ion uptake and energized swelling. When freshwater fishes are subjected to salinity stress, the muscle and gill mitochondrial energy functions are altered in an early response phase (Bashyam *et al*., 1980; Suresh and Jayaraman, 1983). No such changes are observed in the mitochondria isolated from hearts of rats (Davies *et al*., 1981) or dogs (Wollenberger et al., 1961) when subjected to endurance training and gradually induced aortic stenosis, respectively.

As myocardial metabolism is almost exclusively aerobic, the energy required for muscle contraction, ion transport, rhythmicity and for the synthesis of membrane and protein constituents of myocardium are solely supplied by mitochondrial oxidative phosphorylation. In the present system of experimentally induced cardiac hypertrophy in rats produced by constricting the ascending aorta, we have investigated the biogenesis and energetics of mitochondria and the regulatory mechanism governing the process during cardiac hypertrophy. Earlier studies have shown an increase in the mitochondrial biogenesis as early as 24 h after imposition of work load through aortic constriction (Thirunavukkarasu *et al*., 1982). Our results in the present study show an initial response phase at 24 h stage characterized by the altered mitochondrial functions which are restored to normal gradually at the later stages of hypertrophy.

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Abbreviations used: ADP, Adenosine 5 '-diphosphate, ATP, adenosine 5 '-triphosphate; RCI, respiratory control index; TCA, tricarboxylic acid.

Materials and methods

Female albino rats of Wistar derived strain (obtained from Rallis India, Bangalore) were used for the experiments. Animals (150–180 g) were allowed one week to acclimatize to local conditions before aortic constriction was done using a small hemostatic clip (Hemoclip Edward Weck and Company, North Carolina, USA) The animals were fed with Gold Mohur rat feed received from M/s Hindustan Lever Limited, Bangalore, India.

Chemicals

Most of the chemicals used in the experiments were of analar grade obtained from British Drug House and E. Merck, Darmstadt, Germany. Fine chemicals such as adenosine 5' -diphosphate (ADP), adenosine 5' -triphosphate (ATP) etc. were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. $^{45}CaCl_2$ was purchased from the Bhabha Atomic Research Centre, Bombay.

Induction of cardiac hypertrophy

Cardiac hypertrophy was induced by following the method of Rakuson and Poupa (1966) and the percentage hypertrophy was calculated as the percentage increase in the heart weight/body weight ratio of hypertrophic animals over that of the ratio of sham-operated controls (Meenakshi *et al*., 1983).

Isolation of heart mitochondria

Isolation of mitochondria was carried out in a medium containing 250 mM sucrose, 10 mM Tris-HCl, pH 7·5 and 1 mM EDTA. The hearts were homogenized in the isolation medium in a ratio of 1:10. The mitochondria were isolated as described in the earlier paper (Thirunavukkarasu *et al*., 1982). For $^{45}CaCl_2$ uptake and endogenous ion estimation, the mitochondrial pellet was washed 3 times with an EDTA-free isolation medium.

Analytical methods

Protein was estimated according to Lowry et al. (1951) using bovine serum albumin as the standard. Oxygen consumption was measured by polarography (Estabrook, 1967) with a clark type oxygen electrode attached to a recorder. The respiratory control index (RCI) was calculated as the ratio of the rate of oxygen consumption in the presence of ADP (state III) to the rate after the added ADP is completely utilized (state IV). ADP/O ratio was calculated as described earlier (Chance and Williams, 1955).

Endogenous ionic levels

The mitochondrial pellet was washed with EDTA-free isolation medium prepared in deionized and double distilled water. The washed pellet was suspended in 0·05 N HCl. The suspension was kept in boiling water for 30 min and then centrifuged at 10,000 *g*. The clear supernatant fraction was suitably diluted and the ions Na^+ and Ca^{2+} were estimated by flame photometry.

Energy dependent calcium uptake

The procedure adopted for energy-dependent Ca^{2+} uptake by mitochondria was as described by Loyter *et al.*, (1969). The mitochondrial pellet, after washing twice with cold EDTA free medium, was suspended at a concentration of 0·5 mg/ml in the assay medium which contained 150 mM KCl, 4 mM potassium phosphate buffer (pH 7·4), 5 mM Tris-HCl (pH 7·4), 4 mM $MgSO_4$, 5 mM ATP and 10 mM succinate. The reaction was initiated by $^{45}CaCl_2$ and after incubation for 10 min at 30°C, the reaction was stopped by adding 0·1 ml of 400 mM $MgSO_4$. The samples were pelleted by centrifuging at 10,000 *g* for 2 min in a Janetzki T.H. 12 model. After washing the pellet twice with cold assay medium, the pellets were dissolved in 0·1 N NaOH. These samples were then spotted on Whatman No 1 filter paper discs, dried and counted in a liquid scintillation counter.

Mg^{2+}-dependent ATPase

Mg^{2+}-dependent ATPase activity was measured in the mitochondrial preparation according to the procedure of Tzagoloff (1970). The activity was assayed at 30°C in a total volume of 1 ml containing 50 mM Tris-HCl, pH 8·5, 4 mM ATP, 5 Mm $MgSO_4$ and mitochondrial protein of about 70 μg as the enzyme source. The reaction was initiated by the addition of ATP, stopped after 10 min by the addition of 0·5 ml of 10% tricarboxylic acid (TCA), and the tubes were kept at 0°C for 15 min. After pelleting down the precipitate the supernatant fraction was assayed for orthophosphate by the method of Fiske and Subba Row (1952).

Density gradient analysis

For the density gradient analysis a discontinuous sucrose gradient of 30, 35, 40, 45, 50, 60, 65 and 70% (w/v) sucrose was used as described earlier (Suresh and Jayaraman, 1983). Succinate dehydrogenase activity and protein estimations were made in the fractions collected after centrifugation.

Energized swelling

The high amplitude energized swelling of the mitochondria was measured according to the method of Dow et al. (1970). Heart tissue was homogenized and the isolation of mitochondria was carried out using a medium containing 210 mM mannitol, 70 mM sucrose and 1 mM EDTA. About 100 µg mitochondria was added to 1 ml of medium containing 2 mM sodium acetate, 110 mM sodium chloride, 2·5 mM Tris-HCl, pH 7·4, 35 mM mannitol and 0·5 mM EDTA. Swelling was measured as a decrease in absorbance at 520 nm on addition of ATP.

Miscellaneous

The cholesterol content of mitochondria was estimated according to the method of Abell *et al*. (1952) by using Liebermann-Buchard reagent. Extraction of total lipids was carried out following the procedure of Bligh and Dyer (1959). The method of King and Wooton (1959) was followed for the estimation of lipid phosphorus.

Results

Oxidative phosphorylation

A significant increase in succinate oxidation was observed in mitochondria isolated from animals at 24 h after aortic constriction as compared to that of sham-operated. There was no significant change in activity in the mitochondria obtained from animals as early as 6 h after operation or at later stages later than 24 h during developing cardiac hypertrophy. At 24 h post-operative stage the mitochondria exhibited a loss in the ability to phosphorylate the externally added ADP as measured by ADP/O ratio. This function is restored in the mitochondria isolated from hearts at 17, 28 and 40% hypertrophic stages (table 1).

Table 1. Respiratory status of the mitochondria *in vitro* in response to aortic constriction.

Mitochondria	n atoms oxygen uptake/min/mg mitochondrial protein: succinate oxidation	Respiratory control index	Mg^{2+}-dependent ATPase levels (μmol P_i/min/mg protein)
Sham operated	150±30	4·6	0·63±17
6 h after operation	162±12	4·2	0·57±26
24 h after operation	234±42	1·0	0·32±09
17% hypertrophy (2)	174±24	4·0	0·61±12
28% hypertrophy (4)	156±12	4·3	0·69±18
40% hypertrophy (8)	168±12	4·4	0·86±21

2 mg mitochondrial proteins were added to the incubation buffer. 10 mM succinate and 300 nmol of ADP were added to induce respiration. The values in parentheses refer to the number of days after the aortic constriction. The values presented in the table represent the mean of 3 different experiments with ± S.D.

Mg^{2+}-dependent ATPase activity

In order to check whether any alteration occurs in the specific activity of the enzyme located in the inner mitochondrial membrane, Mg^{2+}-dependent ATPase activity was estimated in mitochondria isolated from sham-operated hearts and hearts at different stages of hypertrophy. A slight increase at 6 h post-operative stage and a 2-fold decrease at 24 h after operation in the ATPase activity as compared to that of sham-operated controls were observed. The enzyme activity returned to that of normal values in the later stages of hypertrophy (table 1).

Density gradient analysis

The homogeneity of the mitochondrial preparations from hearts of sham-operated, 24 h post-operative stage and 40% hypertrophic animals, was checked by sucrose density gradient analysis. The mitochondria isolated from hearts of sham-operated and 40% hypertrophic animals showed a major band at 1·2025 g/cm^3. However, the mitochondria from hearts of animals at 24 h post-operative period showed a major band at 1·2296 g/cm^3 and a minor one at 1·2025 g/cm^3 (figure 1).

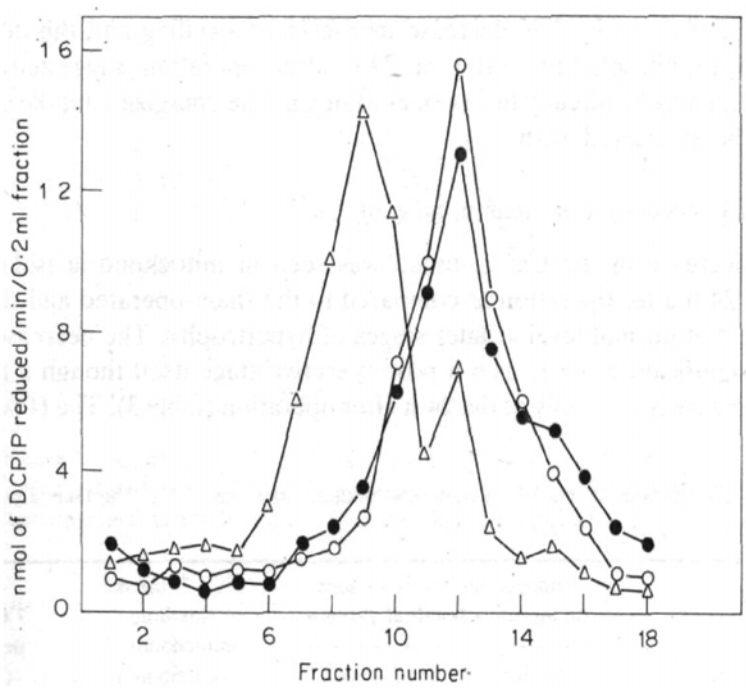

Figure 1. Sucrose density gradient analysis of mitochondria (70–30%).
(O), Mitochondria from sham-operated; (Δ), mitochondria isolated from 24 h after operation; (●), mitochondria from 40% hypertrophic animals.

Energized swelling and cholesterol to phospholipid ratio

A 3-fold decrease in the energized swelling and a 2-fold decrease in cholesterol to phospholipid ratio was observed at 24 h after operation when compared to that of sham-operated controls (table 2). The mitochondria isolated from animals at 6 h after operation did not change very much in the cholesterol to phospholipid ratio but a significant decrease in the energized swelling was seen as compared to the

Table 2 . Quantitative analysis of mitochondrial membrane components and energized swelling rate after aortic constriction.

Mitochondria	Lipid/protein	Cholesterol/phospholipid	Energized swelling Δabsorbance/ min/100 μg mitochondrial protein
Sham operated	0·30±0·09	0.08±0·002	0·107±0·023
16 h after operation	0·25±0·03	0·07±0·004	0.060±0·007
24 h after operation	0.25±0·02	0·04±0·006	0.035±0·003
17% hypertrophy (2)	0·26±0·01	0·06±0·002	0·060±0·009
28% hypertrophy (4)	0·28±0·04	0·08±0·001	0·070±0·013
40% hypertrophy (8)	0·26±0·02	0·08±0·002	0·070±0·015

The values in the parentheses refer to the number of days after aortic constriction. The values presented in the table are the mean of 3 different experiments ± S.D.

sham-operated controls. The decrease in energized swelling and the decrease in cholesterol to phospholipid ratio at 24 h after operation suggested that the mitochondria may be already in a swollen state and the energized uptake of calcium ions may be interfered with.

Endogenous levels of ions and uptake of Ca^{2+}

A 3-fold decrease in the Ca^{2+} uptake was seen in mitochondria isolated from animals at 24 h after operation as compared to the sham-operated and the uptake reached that of normal level at later stages of hypertrophy. The decrease in Ca^{2+} uptake is significant as early as 6 h post-operative stage itself though a maximum level of decrease is seen only at the 24 h after operation (table 3). The 60% increase

Table 3. Endogenous levels of calcium and sodium ions and $^{45}Ca^{2+}$ uptake after aortic constriction.

Mitochondria	Endogenous levels of ions ng/mg mitochondrial protein		$^{45}Ca^{2+}$ uptake nmol/mg mitochondrial protein/min	$^{45}Ca^{2+}$ uptake (%)
	Sodium	Calcium		
Sham operated	29·4±2·3	32·3±2·8	11·3	14·1
6 h after operation	25·5±1·9	39·6±3·1	8·1	10·1
24 h after operation	17·3±0·9	51·1±4·7	4·2	5·3
17% hypertrophy (2)	22·8±0·2	42·2±3·4	9·9	12·3
28% hypertrophy (4)	29·1±2·0	36·8±2·9	9·5	11·9
40% hypertrophy (6)	33·6±2·5	34·1±2·6	10·1	12·6

80 nmol of $^{45}Ca^{2}$ + was added to the reaction mixture to start the reaction for studying the Ca^{2+} uptake. The conditions are as described in the methods section. The values in the parentheses refer to the number of days after aortic constriction. The values presented in the table are the mean of 3 different experiments ± S.D.

in the endogenous calcium levels was accompanied by a 50% decrease in the endogenous sodium levels in the mitochondria obtained from animals at 24 h after operation when compared to that of sham-operated controls. However, the levels of both ions reached that of sham-operated controls gradually in the 17, 28 and 40% hypertrophy (table 3). A slight decrease in endogenous sodium and an increase in the calcium levels were observed in mitochondria at 6 h post-operative stage. The results presented in figure 2 shows an inverse correlation (correlation coefficient – 0.9) between energy dependent ^{45}Ca uptake and the endogenous levels of calcium. These results in addition to the decrease in energized swelling clearly show that alterations in the mitochondrial membrane functions have occurred and these alterations may be reflected in the fluidity of the membrane.

Arrhenius plots

The Arrhenius plots for Mg^{2+}-dependent ATPase activity representing the fluidity of the mitochondrial membranes in the mitochondria isolated from sham-operated hearts and hearts from 24 h post-operative stage and 40% hypertrophy are presented in figure 3. At 24 h after operation, the transition temperature of 18°C,

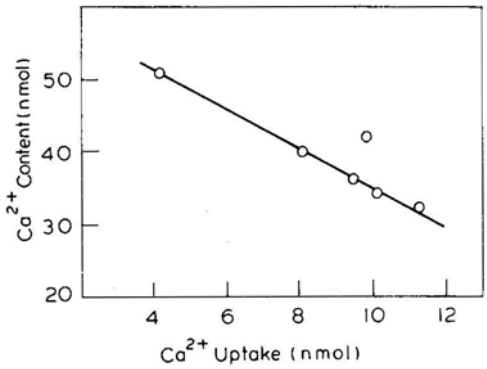

Figure 2. Correlation curve of Ca^{2+} uptake and Ca^{2+} content.

The uptake of $45_{Ca}2+$ and the estimation of calcium content were as given under 'materials and methods' section. The correlation coefficient is $-0{\cdot}9$.

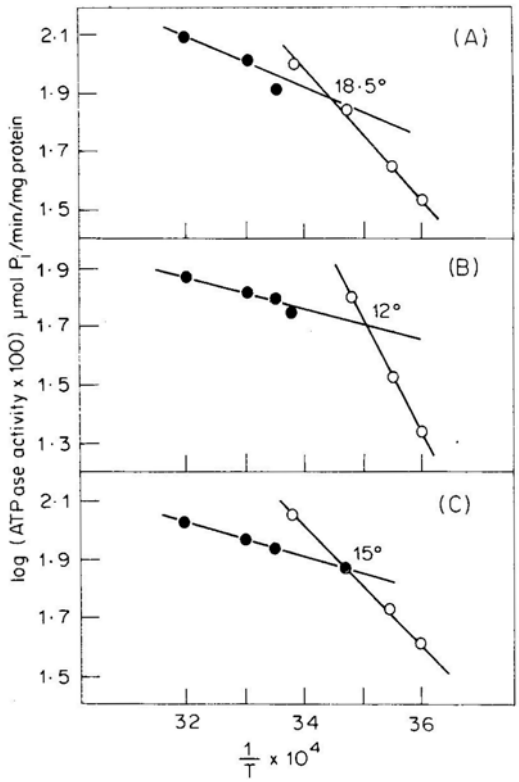

Figure 3. Arrhenius plots of mitochondrial Mg^{2+} dependent ATPase enzyme activity. A. Sham operated controls. B. 24 h after operation. C. 40% hypertrophy.

The transition temperature of the phase change is given in the figure. The activation energy values above the transition temperature are 4·6, 3·2 and 5·4 Kcal/mol in A, B and C, respectively. The activation energy values below the transient temperature are 8·7,12·5 and 6·5 Kcal/mol in A, B and C, respectively.

observed in mitochondria from sham-operated animals, decreased to 12°C, while during 40% hypertrophic stage, it increased to 15°C tending towards the value of sham-operated controls. The activation energy above the transition temperature decreased from 4·6 Kcal/mol in sham-operated to 3·2 Kcal/mol at 24 h after operation and increased to 5·39 Kcal/mol in the 40% hypertrophic stage (figure 3). A reverse trend was seen in the activation energy values below the transient temperature.

Discussion

Calcium is considered to play an important role in the control of metabolic events between mitochondria and cytoplasm in normal cells (Bygrave, 1978). Several reports have attributed the decreased ADP/O ratio, and respiratory control index to the increased loading of calcium in the mitochondria (Rossi and Lehninger, 1964; Villalobo and Lehninger, 1980). Under high salinity stress in fish muscles and gills (Bashyam et al., 1980; Suresh and Jayaraman, 1983) and during muscular dystrophy in hamsters (Mezon et al., 1974), mitochondrial accumulation of calcium ions leads to loss of ADP/O ratio, respiratory control index and uncoupler stimulated respiration.

Our results presented here show a decrease in respiratory control index, ADP/O ratio accompanied by a decreased uptake of calcium ions in mitochondria obtained from aorta-constricted rats at 24 h after operation. A 60% increase in endogenous calcium levels observed at 24 h after aortic constriction may be responsible for the decreased uptake of calcium and associated changes. This view is further strengthened as the respiratory functions and calcium uptake efficiency return to normal levels as the endogenous calcium levels reach that of sham-operated controls at later time points. The shift in the peak of SDH activity to the heavier density region in the density gradient analysis of mitochondria obtained from animals at 24 h after operation as compared to that from sham and 40% hypertrophic animals could be attributed to the increased endogenous calcium levels (figure 1 and table 2).

Influx of calcium ions has been suggested to cause swelling of mitochondria (Carafoli and Lehninger, 1964; Drahota et al., 1965). It remains to be shown whether this reason could be attributed to the decreased energized swelling we have observed in mitochondria from animals at 6 h and 24 h after operation. As mitochondria from sham-operated animals and from hearts at later stages of hypertrophy have lower endogenous levels of calcium ions as compared to that of 24 h stage, the capacity for energized swelling is retained in these mitochondria. The drastic decrease in cholesterol to phospholipid ratio and the corresponding increase in the fluidity of mitochondrial membranes at 24 h post-operative stage could also have contributed to the calcium loading of mitochondria causing decreased swelling at 24 h stage.

Changes in lipid composition of erythrocyte ghosts leading to changes in permeability have been reported in muscular dystrophy (Howland and Iyer, 1977). Also, in ischemia, the liver mitochondria were found to be swollen with loss of respiratory control due to an altered lipid composition (Mittnacht *et al*., 1979). Wollenberger and Schulze (1961) have reported ultra-structural evidence of a

mitochondrial defect in chronically overloaded myocardium of dogs. This information along with our results presented in this paper led us to the presumption that although the stress was caused by different factors, the response by mitochondria usually involved an altered lipid composition leading to a change in membrane configuration. Such a change could cause an influx of calcium into mitochondria resulting in decreased respiratory functions. The transient decrease in the bioenergetic functions during the initial phase of stress and restoration to normalcy later indicate gradual adaptation in the mitochondrial functions at the molecular level to meet the challenge thrown by the imposed stress.

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