ELABORATION OF MEDIUM CHAIN FREE FATTY ACIDS AND LONG CHAIN FATTY ACID PROSTAGLANDIN PRECURSORS BY ISOLATED ANOXIC RAT LIVER MITOCHONDRIA

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Received 10 January 1975

1. Introduction

Mitochondria isolated from ischaemic tissues or 'aged' in vitro exhibit a diminished capacity for oxidative phosphorylation [1–4]. Among the factors implicated has been the liberation of free fatty acids (FFAs) which have been demonstrated to be potent uncouplers of oxidative phosphorylation [5].

A similar deterioration of function was observed during a study of the effects of anoxia upon isolated mitochondria. Determination of liberated FFAs in such experiments revealed an increase not only in medium chain FFAs, but also in polyunsaturated long chain FFAs which are known precursors of the prostaglandins. Since the effects of anoxia have been implicated in cell injury, the elaboration of such prostaglandin precursors may have a role in the cell's response to anoxic injury.

2. Materials and methods

Mitochondria were isolated from the livers of male Sprague-Dawley rats (50–140 g) by the procedure of Weinbach [6]. The isolation medium consisted of 0.25 M sucrose; 10 mM Tris–HCl (pH 7.4); 0.5 mM K-EDTA; 0.5 mM K-EGTA.

A polarographic technique was employed for the production of in vitro anoxia and for the determination of oxidative phosphorylation. Mitochondria were suspended in a medium with a final concentration of 17.9 mM sucrose; 19.3 mM Tris–HCl (pH 7.4); 1.0 mM K-EDTA; 1.0 mM K-EGTA; 111.4 mM KCL; 4.6 mM $MgCl_2$; 4.6 mM K-phosphates (pH 7.4); 20 mM sodium succinate. Mitochondria were present at a level of 1.4–1.7 mg protein/ml.

Anoxia was producted by the exhaustion of O_2 in the polarograph vessel through repeated addition of ADP. When the polarograph recorder trace indicated zero O_2 content in the medium, the mitochondria were maintained in that environment for varying durations. A 3-hour control experiment was also carried out in order to determine the effect of unlimited oxygenation on the experimental system.

Protein was determined by the Biuret method [7]. Mitochondrial lipids were extracted by the method of Garbus et al. [8]. Heptadecanoic acid (C17:O; Sigma) was added to the crude extract as an internal standard to correct for losses during work-up. The solvent phase of the crude extract was collected, evaporated under N_2, and reconstituted in 1.0 ml of chloroform. FFAs were recovered by a modification of the procedure described by Dittmer and Wells [9]. FFAs were esterified using BCl_3–MeOH (10% w/v;

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Applied Sciences Laboratories). The FFA methyl esters were reconstituted in 50-100 μl of hexane (Lipopure; Applied Sciences Laboratories), and used immediately for GLC.

GLC was carried out using a Packard Series 7834 Gas Chromatograph. Packing consisted of 15% EGSS-X with Gas Chrom P (100/120 mesh) as support (Applied Sciences Laboratories). Carrier gas was N_2 at a flow rate of 60 ml/min. Flame ionization detector response was maximized by adjusting H_2 flow. Qualitative and quantitative mixtures of FFA methyl esters were obtained from Sigma Chemical Co., Applied Sciences Laboratories, and Supelco, Inc. The peaks were identified by retention times relative to C 18:0 and by positions of known standards. Quantitation of peak areas was carried out by the triangulation method, the area of C 17:0 serving as the quantitative reference peak.

3. Results and discussion

Oxidative function of mitochondria deprived of O_2 for varying durations versus mitochondria maintained in an aerobic environment for three hours is summarized in table 1. Mitochondria maintained in an anoxic state exhibited an initial decline in State III rate with an increase in State IV rate occurring after two hours of anoxia. This resulted in a continuous deterioration of the RCI, although ADP/O ratios remained relatively intact. After three hours of anoxia there was total loss of the RCI and the ADP/O ratio. In contrast, although mitochondria maintained in an aerobic environment for three hours exhibited an initial decline in respiration in State III, the slight alternation in State IV rates ensured a degree of respiratory control and oxidative phosphorylation.

As it was assumed that the functional deterioration of anoxic mitochondria was a consequence of the elaboration of an endogenous uncoupler, presumably FFAs, extracts of the anoxic mitochondria were analyzed for FFAs by gas-liquid chromatography.

Table 2 shows the concentrations of FFAs recovered after one, two and three hours of mitochondrial anoxia, and the FFAs identified in the three-hour aerated control. The concentrations of all FFAs rise as the anoxic interval is prolonged. This rise seems most apparent in the saturated FFAs, C16:0 and C18:0. The rise in the unsaturated FFAs is progressive but of lesser magnitude than the rise in the saturated FFAs. However, while the concentrations of C 16:0 and C 18:0 after three hr of anoxia are approximately 3–4 times their zero time levels, the concentration of C 20:4 is approximately 24 times its zero time value. Furthermore, after three hr of anoxia the ratio of total saturated FFAs to total unsaturated FFAs approximates 1:1. It is readily apparent that the concentration of FFAs recovered in the three-hour control is much below the concentration of FFAs in the zero time control.

In the present study we have demonstrated that mitochondria rendered anoxic in a polarographic medium elaborate increasing amounts of FFAs as is found with mitochondria 'aged' in vitro or in

Table 1
Oxidative function of anoxic mitochondria compared to aerobic mitochondria

Anoxic				Aerobic			
Time (hr)	State III + State IV" nAO_2/min/mg	RCI*	ADP/O	Time (hr)	State III + State IV" nAO_2/min/mg	RCI*	ADP/O
0	87.29	15.38	5.76	2.41	0	87.11	13.21	6.59	2.21
1	32.99	11.94	2.76	2.33	1	21.78	9.32	2.34	2.66
2	42.17	30.08	1.40	2.19	2	21.78	13.93	1.56	2.51
3	39.63	39.63	1.00	0.00	3	22.49	14.28	1.57	1.57

After the anoxic period, portions of the mitochondrial suspensions were re-oxygenated, and their function determined polarographically. The control consisted of a suspension of mitochondria which was continuously exposed to the atmosphere.
+ State III – rate of oxidation in the presence of ADP and substrate
" State IV – rate of oxidation in the presence of substrate after the exhaustion of added ADP
* RCI – respiratory control index; the ratio of State III to State IV rates

Table 2
Concentration of free fatty acids under various conditions*

Free fatty acid	Control (O time)	One hr Anoxia	Two hr Anoxia	Three hr Anoxia	Three hr Control+
C16:0	1.68	3.25	5.09	5.19	0.76
C18:0	1.15	4.09	6.91	3.74	0.32
C18:1	0.85	1.28	1.68	3.23	0.29
C18:2	0.44	1.23	2.10	2.22	0.05
C20:2	0.22	0.08	0.15	0.25	0.06
C20:4	0.08	0.55	1.39	1.87	0.02
C20:5	0.00	0.16	0.35	0.25	0.00
C22:6	0.00	0.11	0.21	0.19	0.00
Total	4.42	10.75	17.88	16.94	1.50

FFAs were extracted and quantified by GLC as described in Materials and methods.
* Expressed as nmol of free acid per milligram of mitochondrial protein.
+ FFAs recovered *after* 3 hr under aerated conditions.

mitochondria isolated from ischaemic tissue. Concomitant with this rise in FFA concentration, deterioration of oxidative function is observed. However, if mitochondria are maintained in an oxygenated medium for similar periods of time, there is no accumulation of FFAs, and oxidative function is relatively intact.

The mechanisms by which anoxic mitochondria or mitochondria isolated from ischaemic tissue or aged in vitro accumulate FFAs can be attributed to the activity of lipases [2–4] and the inhibition of oxidation, either directly or by inhibition of the fatty acid activating system. As mitochondrial function deteriorates as a consequence of anoxia, sequestered Ca^{2+} could be released activating phospholipases and the liberated FFAs would accumulate due to the inhibition of β-oxidation.

In the present study, the initial rise in FFAs was largely due to saturated FFAs even though these saturated fatty acids acount for only 33% of the total mitochondrial fatty acids [10]. Of more interest is the proportionately greater increase in the polyunsaturated FFAs C 20:4, C 20:5 and C 22:6. Also, the fatty acids, C 20:2, C 20:5 and C 22:6 which were found in mitochondria rendered anoxic for 1–3 hr were nor previously reported in aged mitochondria [3], nor in mitochondria isolated from ischaemic liver [4]. These longer chain polyunsaturated fatty acids are known precursors of prostaglandins of the E and F series [11]. As the liver has been shown to possess the enzymatic complement needed for prostaglandin synthesis [12], the accumulation of these known prostaglandin precursors may represent the penultimate step for synthesis of prostaglandins.

The synthesis and release of prostaglandins may represent a mechanism by which the cell deals with the deleterious effect of anoxia. Support for this hypothesis comes from studies demonstrating increased levels of prostaglandin released from tissues rendered ischaemic [13–18]. Furthermore, unpublished observations in this laboratory have shown an increase with time in endogenous FFAs in various organs rendered anoxic that parallel the organs' susceptibility to ischaemic damage.

That mitochondria may be the regulators of the levels of prostagladins during ischaemic episodes in intact cells is an intriguing concept. The mechanisms through which mitochondria may provide precursors for prostaglandins in ischaemia have been discussed above. When the ischaemic episode ceases and an increased level of O_2 is available to the mitochondria β-oxidation of accumulated FFAs would be stimulated, Ca^{2+} resequestered, and endogenous prostaglandins metabolized by mitochondria as described by Hamberg [19]. The shift in the $NAD^+/NADH$ ratio would also enhance the activity of the cytoplasmic prostaglandin degradation system [20,21].

This proposed mitochondrial control of prostaglandin levels would have a teleological purpose. The

synthesis of prostaglandins from elaborated FFAs not only would protect the cell from the deleterious effects of uncoupling, but also would provide a potent messenger to signal the local environment and the systemic circulation of the ischaemic cell's plight. With the restoration of the availability of O_2, mitochondria would function to lower the levels of intracellular prostaglandin, and thus effectively counter its role as a messenger.

Acknowledgements

The authors wish to express their thanks to Mr Robert Seamon for technical assistance, and to Mrs Gloria Taylor and Mrs Constance Kouns for editorial assistance.

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