THE PRESENCE OF THE GLYCEROL PHOSPHATE SHUTTLE AND ENERGY DEPENDENT TRANSHYDROGENASE IN AORTIC MITOCHONDRIA

Vijay K. Kalra and Arnold F. Brodie

From the Department of Biochemistry
University of Southern California School of Medicine
Los Angeles, California 90033

Received February 8, 1973

SUMMARY. A procedure has been devised for isolating tightly coupled mitochondria from the intima-media strip of pig aorta. The mitochondria exhibit respiratory control with succinate, pyruvate-malate, β-hydroxybutyrate or α-glycerol phosphate as substrate. Furthermore, submitochondrial particles from these preparations exhibit an energy-dependent reduction of $NADP^+$ by NADH.

INTRODUCTION. Although a vast amount of information is available on the metabolism of heart tissue, less is known about the means by which other vascular tissues obtain energy for biosynthesis, repair, contraction or transport of foodstuff. Studies with arterial tissues susceptible to atherosclerosis revealed that enzymes of the tricarboxylic acid cycle decreased in the diseased state (1). It has been suggested by Whereat (2) that aortic mitochondria may be uncoupled or damaged in the diseased state. Very little is known about the oxidative metabolism of the intima-media of the aorta because of the difficulty in isolating undamaged mitochondria. One major problem is that the smooth muscle cells of the arteries are enclosed in collagen and elastin. Recently Morrison et al., (3) have been able to isolate mitochondria from the intima-media of pig aortic tissue by treating with elastase. The mitochondria exhibited respiratory control with succinate, pyruvate-malate or glutamate as substrate.

In the present study, a procedure has been developed for the isolation of aortic mitochondria without the use of enzymes. The mitochondria were examined for the presence of the α-glycerol phosphate shuttle and the energy-linked (ATP) transhydrogenase reaction. These two reactions of energy metabolism may possibly be regulated or involved in the endogenous synthesis of fatty acids and triglycerides in the wall of the aorta.

MATERIALS AND METHODS. Piglets (male and female, 4-6 weeks old, weighing between 12-15 Kg) were fed commercial pig chow and water. They were euthanized by embolism or by a captive bolt gun and then exsanguinated. The aorta (thoracic and abdominal) was removed and placed in an isolation medium which consisted of 100 mM KCl, 10 mM EDTA, 25 mM Tris-HCl buffer (pH 7.4) and 0.5% bovine serum albumin (fatty acid-poor fraction V). The aorta was cut open, adventia removed, and the orfices of all blood vessels of the aorta were removed with a cork borer or TECNA Aortic Punch (Tecna Corp., Emeryville, CA). The intima and the adjacent media layers were removed from the aorta with forceps and placed in the isolation medium (2-4°).

Preparation of Mitochondria - The intima-media was suspended in 5-6 volumes of isolation medium and cut into small pieces with a scissors and subsequently homogenized. Homogenization of the tissue was performed with either a Polytron homogenizer while flushing with argon or a Virtis homogenizer 60 for 15 sec at 20,000 rpm using a macroshaft assembly at 2-4°. The homogenate was filtered through one layer of cheesecloth; the residual tissue was resuspended in 5-6 volumes of isolation medium and rehomogenized by the procedure described above. The filtrates were combined and centrifuged twice at 700 g for 15 min to remove nuclei and debris. The resulting supernatant was centrifuged twice at 8000 g for 10 min to yield a mitochondrial pellet which was resuspended in 1.0 - 2.0 ml of assay medium containing 0.5 M Mannitol, 40 mM KCl, 10 mM K-phosphate buffer (pH 7.2), 6 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.2).

Respiratory Control Studies - Respiration of mitochondria (0.25 - 0.30 mg protein) in state 3 and state 4 was measured at 30° in a total volume of 1.0 ml using a Clark oxygen electrode (4). Acceptor control ratios and ADP/O ratios were determined by the method of Chance (5) and Estabrook (6). Respiration studies were also performed in the presence of exogenous horse heart cytochrome c (0.2 - 0.3 mg). The cytochrome c was added to the reaction mixture since it was observed that there was a loss of endogenous cytochrome c during the homogenization in the salt medium in some of the mitochondrial preparations.

Energy-Dependent Reduction of $NADP^+$ by NADH - Submitochondrial particles were prepared by the procedure of Low and Vallin (7). The energy-linked transhydrogenation reaction of aortic submitochondrial particles was assayed by the procedure described by Danielson and Ernster (8). Protein was determined by the method of Lowry et al., (9).

RESULTS AND DISCUSSION. In the proliferative phase of atherosclerosis it has been observed that there is an increase in the oxygen uptake of aortic tissue (10, 11). This increase may be due to uncoupling of oxidative phosphorylation of mitochondria (12). However, studies with rat aortic mitochondria have shown that there is no impairment of oxidative phosphorylation using pyruvate-malate as a substrate during experimentally induced atherosclerosis (13). In the present study, mitochondria were isolated from the intima-media of aortic tissue and examined for the α-glycerol phosphate shuttle and the energy-linked transhydrogenase reaction. The mitochondria were obtained from aortic tissue with either a Polytron homogenizer or a Virtis 60 homogenizer. This eliminated the need for predigestion with elastase. Argon was flushed into the reaction mixture because dissolved oxygen in the isolation medium may cause cavitation of the mitochondria and render them susceptible to disruption. Homogenization of the aortic tissue in Chappel-Perry medium (0.1 M KCl, 0.05 M Tris-HCl buffer, pH 7.4, 0.001 M Na-ATP, 0.005 M $MgSO_4$ and 0.001 M EDTA) resulted in a partial or complete release of cytochrome c. In these studies, the addition of exogenous cytochrome c to mitochondria was necessary to observe high respiratory control. The mitochondrial preparations contained cytochromes b and $a + a_3$, but in general lacked cytochrome c, which had leaked into the medium. The loss of cytochrome c during isolation is not uncommon with mitochondria from other tissues (14).

Respiratory Control and Oxidative Phosphorylation - Mitochondria from pig aorta were examined for the ability to utilize different substrates for demonstrating respiratory control. In agreement with the findings of Morrison et al., (3) the mitochondria exhibited respiratory control (ratio of state 3 respiration/state 4 respiration) with succinate or pyruvate-malate (Table I). Furthermore, respiratory control was also observed with α-glycerol phosphate and β-hydroxybutyrate (Table I). In addition

TABLE I

Oxidative Phosphorylation in Pig Aorta Mitochondria

Substrate	No. of Studies	State 3 Respiration	ACR	ADP/O Ratio
Succinate	6	1.18 ± 0.21	2.6 ± 0.4	1.68 ± 0.17
α-Glycerol phosphate	6	0.48 ± 0.10	2.4 ± 0.6	1.4 ± 0.4
β-Hydroxy-butyrate	3	1.68 ± 0.20	2.5 ± 0.3	2.4 ± 0.2
Pyruvate-malate	3	0.80 ± 0.25	2.9 ± 0.3	2.5 ± 0.3

State 3 respiration: μmoles O_2/hr/mg protein. The reaction mixture for assay contained mitochondrial protein (0.2 - 0.4 mg protein), 0.225 M Mannitol, 20 mM KCl, 5.0 mM K-phosphate buffer (pH 7.2), 3 mM $MgCl_2$. Substrates were added at a final concentration of 10 mM. After the initial rate was established with the substrate, 0.02 - 0.03 mM ADP was added. The temperature for all assays was 30°. ACR refers to acceptor control ratios.

TABLE II

Effect of Inhibitors on Oxidative Phosphorylation in Aorta Mitochondria

With α-Glycerol Phosphate as Substrate

Inhibitor		No. of Studies	State 3 Respiration
None		4	1.18 ± 0.21
Antimycin A	2 μg/mg protein	4	0.0
KCN	(2×10^{-3}M)	4	0.08
DNP	(2×10^{-5}M)	4	1.68 ± 0.12

Conditions for the assays were the same as described in the legend for Table I.

respiration with α-glycerol phosphate was inhibited by Antimycin A or KCN and uncoupled by DNP (Table II). Although cytoplasmic NADH cannot penetrate mitochondrial membrane, electrons derived from it can enter the mitochondrial electron transport chain by indirect routes; e.g., α-glycerol phosphate shuttle.

α-Glycerol phosphate metabolism has been studied in obese patients. It was

observed (15) that α-glycerol phosphate dehydrogenase activity of both the cytoplasm and mitochondria in adipose tissue was diminished in severely obese patients when compared to slender control subjects. The conclusion was that this diminished activity promoted lipogenesis by increasing the α-glycerol phosphate available for conversion to glycerol and triglycerides. It is possible that the α-glycerol phosphate shuttle is inoperative in cells of diseased arteries even though the tricarboxylic acid cycle and electron transport proceed at normal aerobic rates. Preliminary studies (16) have shown an accumulation of high levels of α-glycerol phosphate in stressed pigs and in atherosclerotic susceptible pigeons.

Energy-Linked Transhydrogenase - Various aspects of the energy-dependent reduction of $NADP^+$ by NADH have been studied with submitochondrial particles from both rat liver and beef heart (8, 17). However, no attempt has been made to ascertain whether the energy-dependent transhydrogenase is existent in the submitochondrial particles derived from aortic tissue. The energy-dependent (ATP) reduction of $NADP^+$ by NADH was found to occur in pig aorta submitochondrial particles (Fig. 1). The rate of reduction of $NADP^+$ was found to proceed at a linear rate upon the addition of ATP and was inhibited by thyroxine and tetraacetyl thyroxine. In the absence of ATP, the addition of $NADP^+$ to the NADH generating system resulted in only a small increase in absorption at 340 nm. An increase in absorption at 340 nm did not occur when NADH was omitted. Results analogous to those shown in Fig. 1 were obtained when either rotenone or Antimycin A were used, instead of KCN, to block the aerobic oxidation of NADH.

NADPH/$NADP^+$ levels in cells can control or regulate the synthesis of triglycerides and fatty acids. It is possible that the transhydrogenase reaction may partially control the accumulation of fatty acids and triglycerides in diseased arteries.

ACKNOWLEDGMENTS. This work is part of the project on "Specialized Center for Research-Atherosclerosis" NHLI Grant 14138. The authors would like to express their deep appreciation to Dr. David H. Blankenhorn for valuable discussions, encouragement and his help in making these studies possible. We acknowledge the advice of Drs. E. S. Morrison and R. F. Scott regarding the use of the Virtis homogenizer for the preparation of mitochondria and also the technical assistance of Otokar Brezina and Charles Maier.

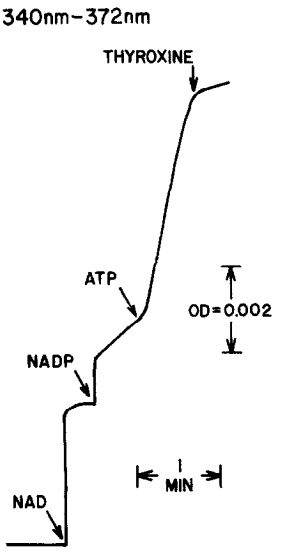

Figure 1. ATP-Dependent Reduction of $NADP^+$ by NADH.

The reaction mixture consisted of 50 mM Tris buffer (pH 8.0), 6.0 mM $MgCl_2$, 250 mM sucrose, 1 mM KCN, 60 mM ethanol, 0.25 mg of alcohol dehydrogenase and piglet aorta submitochondrial particles (1.48 mg of protein). Further additions were: 0.017 mM NAD^+, 0.2 mM $NADP^+$, 1 mM ATP, 4 μg thyroxine. The final volume was 2 ml and the temperature was 26°. The reaction was followed with a dual-wavelength spectrophotometer (American Instrument Co.).

REFERENCES

1. Zemplenyi, T., "Enzyme Biochemistry of the Arterial Wall as Related to Atherosclerosis," London, Lloyd-Luke, 1968.

2. Whereat, A.F., J. Atheroscler. Res., 4, 272, 1964.

3. Morrison, E.S., Scott, R.F., Kroms, M. and Pastori, S.J., Biochem. Med., 4, 47, 1970.

4. Chance, B. and Williams, C.R., Nature, 175, 1120, 1955.

5. Chance, B., Ciba Found. Symp., Regulation Cell Metabolism, pp. 91, Little, Brown, Boston, Mass., 1959.

6. Estabrook, R.W., in "Methods in Enzymology," Vol. X, (R.W. Estabrook and M. E. Pullman, ed.), pp. 41, Academic Press, New York and London, 1967.

7. Low, H. and Vallin, I., Biochim. Biophys. Acta, 69, 361, 1963.

8. Danielson, L. and Ernster, L., Biochem. Z., 338, 188, 1963.

9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem., 193, 265, 1951.

10. Whereat, A.F., J. Lipid Res., 7, 671, 1966.

11. Scott, R.F., Morrison, E.S. and Kroms, M., J. Atheroscler. Res., 9, 5, 1969.

12. Murray, M., Schrodt, G.R. and Berg, H.F., Biochem. Med., 2, 118, 1968.

13. Simard-Duquesne, N., J. Atheroscler. Res., 10, 97, 1969.

14. Hedman, R., Exptl. Cell Res., 38, 1, 1965.

15. Galton, D.J. and Bray, G.A., J. Clin. Endocrinol. Metab., 27, 1573, 1967.

16. Kalra, V.K. and Brodie, A.F., in preparation.

17. Ernster, L. and Lee, C.P., Ann. Rev. Biochem., 33, 729, 1964.