MITOCHONDRIAL FUNCTION IN THE ATRIAL APPENDAGE OF THE HUMAN HEART. CHARACTERIZATION BY HIGH-RESOLUTION RESPIROMETRY

Frank N. Gellerich[a], **Rosmarie Steinlechner**[a], **Markus Wyss**[a], **Thomas Eberl**[a], **Ludwig C. Müller**[b], **Daniela Skladal**[c], **Wolfgang Sperl**[c], **Otto Dapunt**[b], **Raimund Margreiter**[a], **Erich Gnaiger**[a]

[a]*Department of Transplant Surgery, Clinical and Interdisciplinary Bioenergetics;* [b]*Department of Heart Surgery;* [c]*Department of Pediatrics, University Hospital of Innsbruck, Anichstr. 35, A-6020 Innsbruck, Austria. E-mail: erich.gnaiger@uibk.ac.at*

INTRODUCTION

There is an increasing number of reports on biochemical analyses of human biopsy samples to detect bioenergetic defects responsible for mitochondrial cytopathies [1-4] and acute cell injury [5,6]. Controversial reports on changes of mitochondrial enzyme activities in myopathic hearts exist [2,3]. Therefore, in addition to measurements of single mitochondrial enzymes, functional investigations of mitochondria are necessary to detect and quantify mitochondrial defects. For investigations on the correlation of changes in enzyme activities with altered mitochondrial function, the normal state of human heart mitochondria has to be defined. So far, isolation of mitochondria and subsequent polarographic investigations have been used as the state of the art procedure [1]. As an alternative, the technique of permeabilization of muscle fibers has been introduced for characterization of mitochondrial function [7-10]. This method is advantageous for several reasons: *(1)* The amount of tissue required for functional investigations can be reduced to about 30 mg wet weight [4]. *(2)* Mitochondria remain in a more physiological surrounding in comparison to isolated mitochondria. *(3)* The long-term stability of dissected muscle fibers is remarkable [10] in High Energy Preservation Solution (HEPS) [7].

In addition, experimental developments of high-resolution respirometry [11] and the multiple substrate-inhibitor titration techniques [12] allow functional investigation of human tissue specimens obtained by needle biopsy [12].

In the present study, we characterized the mitochondrial function in skinned fibers of the human right atrial appendage. Removal of the atrial appendage prior to cannulation of the atrium is an integral part of coronary artery bypass operations. Tissue samples were obtained for bioenergetic diagnosis from five patients with coronary artery diseases undergoing bypass grafting without atrial rhythm disturbances. The method is aimed at a characterization of chronical or acute disturbances of energy metabolism in the human heart, to improve the diagnosis of cardiomyopathies and to yield a quantitative measure of mitochondrial damage during ischemia and reperfusion.

METHODS

Storage and skinning of fibers: Specimens of the right cardiac atrial appendage were immediately put into ice cold storage medium containing high amounts of ATP and creatine phosphate as energy supplying substances (HEPS [7]; 10 mM EGTA-CaEGTA buffer, free Ca^{2+} concentration 0.1 µM, 9.5 mM $MgCl_2$,

3 mM KH_2PO_4, 20 mM taurine, 5 mM ATP, 15 mM phosphocreatine, 49 mM K-MES and 29 mM imidazole-HCl, pH 7.1). For dissection with small needles, the fibers were put into a droplet of HEPS on a petri dish. Small bundles of fibers were added to 1 ml HEPS containing additionally 50 µg/ml saponin and gently mixed for 30 min at 4 °C. The fibers were washed three times in incubation medium (75 mM mannitol, 225 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 0.5 mM Na_2EDTA, 5 mM $MgCl_2$, 1 mg/ml BSA, 20 mM Tris-HCl, pH 7.4).

Respirometry: Fibers were weighed on a microbalance after removing the adherent liquid by a filter paper. Bundles of about 6 mg wet weight were transferred into the OROBOROS® *Oxygraph* (PAAR, Graz, Austria) [11]. The chamber volumes were set at 2 ml and measurements were performed in incubation medium at 30 °C. The oxygen concentration at air saturation of the medium was considered to be 200 nmol O_2/ml at 95 kPa barometric pressure. The weight-specific oxygen consumption rates [nmol O_2/(s·mg wet weight)] were calculated as the time derivative of the oxygraph trace (DATGRAF 2.2 Analysis Software, OROBOROS®).

RESULTS AND DISCUSSION

In order to obtain a rapid overview on the mitochondrial function in a well-defined part of the human heart, we developed an experimental protocol allowing us to obtain all important information within a short time after removal of the tissue. About 25 mg of permeabilized fibers of patients without cardiomyopathies were distributed among four oxygraph chambers of two instruments operated simultaneously. In each chamber, a different substrate was used (10 mM pyruvate and 2 mM malate; 1 mM octanoylcarnitine and 2 mM malate; 100 µM palmitoylcarnitine and 2 mM malate, 10 mM succinate and 10 µM rotenone). A typical oxygraph experiment with 10 mM pyruvate and 2 mM malate is shown in Fig. 1. The thick line (left axis) represents the original oxygen-time curve from which the first derivative was calculated (thin line, right axis). After addition of the fiber into incubation medium, substrate-dependent endogenous respiration (without added ADP and ATP) was measured. Subsequently 1 mM ADP was added to obtain active respiration (state 3). This high ADP concentration was necessary to overcome the remarkable concentration gradient between the bulk phase and the mitochondria within the fibers. State 4 respiration was measured after addition of 50 µM atractylate, an inhibitor of the ATP/ADP-translocator. From the state 3/state 4 ratio of respiratory rates the respiratory control index (RCI) was calculated (3.3 in this example). Subsequently uncoupled respiration was initiated by addition of FCCP, an uncoupler of oxidative phosphorylation. The uncoupled rate was in this experiment higher than state 3 respiration, indicating that the respiratory chain has a slightly higher capacity than the phosphorylation under these conditions. Finally the respiratory chain was inhibited by addition of 13 µM antimycin A, an inhibitor of the *bc*1-complex (complex III). Even at an exceeding concentration of antimycin A, inhibition of the respiratorty chain is not complete until about 15 min (Fig. 1). Finally, a few mg of dithionite were added for zero calibration of oxygen.

The results obtained from 9 to 19 single incubations of 5 different hearts are presented in Table 1. State 3 respiration (11.5 to 16.3 pmol O_2/(s·mg w.w.)] was highest with palmitoylcarnitine as substrate followed by succinate, pyruvate and

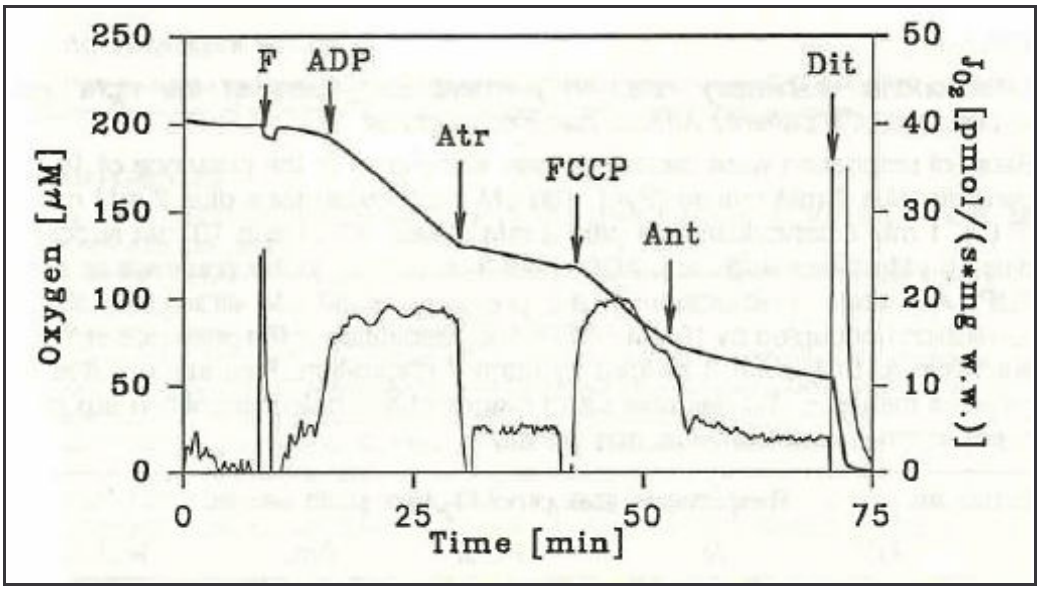

Fig. 1. Typical oxygraph traces of mitochondrial respiration in permeabilized fibers of a human right atrial appendage. Incubation with 10 mM pyruvate and 2 mM malate as substrates. Additions: F, 7.5 mg fibres; ADP, 1 mM ADP; Atr, 50 µM atractylate; FCCP, 10 µM FCCP; Ant, 13 µM antimycin A; Dit; a few mg of dithionite. Measurement of oxygen concentration in an *Oxygraph* (left axis). The (negative) time derivative of this signal indicates the rate of respiration (right axis).

octanoylcarnitine. It is interesting that these rates of respiration were lower than those measured in human *m. vastus lateralis* (13.3; 14.7; 18.3; and 21.8 pmol $O_2 \cdot s^{-1} mg^{-1}$ w.w. for palmitoylcarnitine, octanoylcarnitine, succinate and pyruvate as substrates, respectively). A lower mitochondrial content in muscle fibers of the human atrial appendage might be responsible for these differences. Heart fibers oxidize palmitoylcarnitine with the highest rate, corresponding well with the enzyme pattern and the metabolic demands of this tissue.

The RCI was highest (3.3 ± 0.9) with pyruvate/malate as substrates and remarkably lower with succinate (2.1 ± 0.6.). This difference is probably caused by the higher state 4 respiration in the presence of succinate due to the lower H^+/O stoichiometry of succinate in comparison to NAD-dependent substrates. For the same reason, the succinate-induced leak respiration (atractylate minus antimycin A respiration) is higher for succinate than for the other substrates. The high state 4 respiration with palmitoylcarnitine may be explained by an uncoupling effect of this substrate.

On average, uncoupled rates of respiration were slightly lower than the state 3 respiratory rates, although in several experiments higher uncoupled rates were observed (*e.g.* Fig. 1). This can be explained by the well-known concentration dependence of uncoupling and inhibiting actions of uncouplers. Therefore, the optimum concentration is different in each incubation depending on the mitochondrial content of fiber.

TABLE 1

Mitochondrial respiratory rates in permeabilized fibers of the right atrial appendage of 5 patients without cardiomyopathies

Rates of respiration were measured (see also Fig. 1) in the presence of 10 mM pyruvate plus 2 mM malate (Pyr.), 100 µM palmitoylcarnitine plus 2 mM malate (P.C.), 1 mM octanoylcarnitine plus 2 mM malate (O.C.) and 10 mM succinate plus 10 µM rotenone (Succ.). ADP, state 3 respiration in the presence of 1 mM ADP; Atr., state 4-respiration in the presence of 50 µM atractylate; FCCP, respiration uncoupled by 10 µM FCCP; Ant., respiration in the presence of 13 µM antimycin A; RCI, state 3 divided by state 4 respiration. Respiratory rates are given as means ± S.D. Numbers and ranges of all single incubations are given in parentheses and brackets, respectively.

Substrate	Respiratory rates pmol O_2/(s·mg wet weight)				
	ADP	Atr.	FCCP	Ant.	RCI
Pyr.	12.6±2.8 (18)	4.1±1.3 (19)	10.5±2.4 (12)	3.4±1.7 (19)	3.3±0.9 (19)
	8.1 - 18.6	2.0 - 7.4	7.0 - 12.9	0.6 - 6.6	1.8 - 5.3
P.C.	16.3±2.0 (9)	6.7±1.1 (9)	13.7±1.3 (8)	4.9±2.1 (8)	2.5±0.5 (9)
	13.3 - 18.0	5.0 - 9.0	11.7 - 16.0	2.6 - 8.8	2.0 - 3.4
O.C.	11.5±2.8 (8)	4.1±1.3 (9)	8.9±3.9 (7)	3.1±1.1 (9)	2.7±0.5 (9)
	6.6 - 14.9	2.2 - 5.9	6.8 - 16.4	1.6 - 4.6	2.0 - 3.4
Suc.	15.0±3.7 (9)	7.2±2.0 (9)	12.7±2.4 (8)	3.9±0.9 (9)	2.1±0.6 (9)
	7.4 - 19.9	4.5 - 10.6	8.8 - 16.4	2.5 - 5.1	1.5 - 3.4

The data in Table 1 represent the means of all single incubations, combining the variation between single fibers of an individual patient and the variation between different patients. The variation between the individual patients was much lower (12.5 ± 1.3 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ w.w., n=5, range 10.6 - 14.4 for pyruvate as substrate). The reason is probably the microheterogeneity of mitochondrial concentrations between single fibers of a patient. Similar results were observed for human skeletal muscle [4]. Each substrate should, therefore, be measured in at least three different fibers to reduce the effect of microheterogeneity.

CONCLUSION

The functional properties of mitochondria in the right atrial appendage of patients are amenable for routine diagnosis, using permeabilized fibers and high resolution respirometry. Further investigations are needed for a comparison of atrial and ventricular myocardium to evaluate the relevance of atrial tissue samples for the clinical state of the human heart.

ACKNOWLEDGEMENTS

This work was supported by the University of Innsbruck and the DFG (Ge 664/7-1). F.N.G. thanks the University of Innsbruck for the possibility to work as a visiting professor.

REFERENCES

1 Rustin P, Chretien D, Bourgeron T, Gerard B, Röting A, Saudubray JM, Munnich A (1994) *Clin Chim Acta* **228**: 35-51

2 Buchwald A, Till H, Unterberg C, Oberschmidt R, Figulla HR, Wiegand V (1990) *Eur Heart J* **11**: 509-516

3 Maurer I, Zierz S (1993) *Am J Cardiol* **72**: 428-433

4 Gellerich FN, Skladal D, Schranzhofer R, Lanznaster N, Kunz WS, Wisniewski E, Kuznetsov A, Krismer M, Gnaiger E, Margreiter R, Sperl W. (1994) In *Molekulare Grundlagen hereditärer Myopathien* (Gross M, Gresser, U, eds) W Zuckerschwerdt Verlag München

5 Bretschneider HJ, Hubner G, Knoll D, Lohr B, Nordbeck H, Spieckermann PG (1975) *J Cardiovasc Surg* **16**: 241-260

6 Snyder JW, Pastorino JG, Hoek JB, Farber JI (1993) *Am J Physiol* **264**: C709-C714

7 Veksler VI, Kuznetsov AV, Sharov VG, Kapelko VI, Saks VA (1987) *Biochim Biophys Acta* **892**: 191-196

8 Letellier T, Malgat M, Coquet M, Moretto B, Parrot-Roulaud F, Mazat JP (1992) *Pediatr Res* **32**: 17-22

9 Kunz WS, Kuznetsov AV, Schulze W, Eichhorn K, Schild L, Striggow F, Bohnensack R, Neuhof S, Grasshoff H, Neumann HW, Gellerich FN (1993) *Biochim Biophys Acta* **1144**: 46-53

10 Skladal D, Schranzhofer R, Krismer M, Sperl W, Eberl T, Margreiter R, Gnaiger E, Gellerich FN (1994) In *What is Controlling Life?* (Gnaiger E, Gellerich FN, Wyss M, eds) *Modern Trends in BioThermoKinetics* **3**, Innsbruck Univ Press: 268-271

11 Haller T, Ortner M, Gnaiger E (1994) *Anal Biochem* **218**: 338-342

12 Gellerich FN, Skaldal D, Lanznaster N, Sperl W, Schranzhofer R, Krismer M, Steinlechner R, Margreiter R, Gnaiger E (submitted)