Biochimica et Biophysica Acta, 1141 (1993) 90–94

BBABIO 43747

Relationships between age-dependent changes in the effect of almitrine on H^+-ATPase/ATPsynthase and the pattern of membrane fatty acid composition

Muriel Jumelle-Laclau [a], Michel Rigoulet [a], Nicole Averet [a], Xavier Leverve [b], Liliane Dubourg [c], Annette Carbonneau [c], Michel Clerc [c] and Bernard Guerin [a]

[a] *Institut de Biochimie cellulaire et Neurochimie du CNRS, Université de Bordeaux II, Bordeaux (France),* [b] *Laboratoire de Thérapeutique, Université Joseph Fourier, Grenoble (France) and* [c] *Laboratoire de Biochimie Médicale A, Université de Bordeaux II, Bordeaux (France)*

(Received 15 April 1992)

Key words: ATPase/ATPsynthase; Almitrine; Oxidative phosphorylation; Lipid composition; Age; (Rat liver mitochondrion)

The effects of almitrine on ATPase/ATPsynthase previously described in beef heart mitochondria (Rigoulet et al. (1990) Biochim. Biophys. Acta 1018, 91–97) are also observed in liver mitochondria isolated from rats older than 7 weeks. In contrast, in rats younger than 5 weeks, almitrine at the same concentration has no effect on the ATPase/ATPsynthase complex. This age-dependent action of almitrine is well correlated with age-dependent modifications of two fatty acids: linoleic and docosahexaenoic acids. The possibility of a change in H^+/ATP stoichiometry of the ATPase/ATPsynthase induced by almitrine seems related to more general modifications of membrane properties during growth of the rat.

Introduction

We have previously described the mode of action of almitrine in three kinds of mitochondria isolated from yeast, bovine heart and rat liver [1–3]. In all the cases, (i) almitrine inhibits ATPase activity and decreases the ATP/O ratio of oxidative phosphorylation without any change in the degree of protonmotive force; (ii) the higher the ATP synthesis and respiratory fluxes, the larger the decrease in the ATP/O ratio induced by almitrine; (iii) almitrine has no direct effect on the respiratory chain; (iv) almitrine increases the quantity of both potassium acetate and potassium phosphate accumulation sustained by ATP utilisation, and also the electrical charge/ATP ratio at steady state ATPase activity. From all these results, we have proposed that almitrine increases the mechanistic stoichiometry of ATPase/ATPsynthase and that the inhibitory effect of almitrine on this complex is only the consequence of such an H^+/ATP stoichiometry change.

In investigating the flow-force relationships in rat liver mitochondria, we have observed different situations dependent on the age of the rat used [4]. For instance, the relationship between protonmotive force and respiratory rate was the same whatever the way in which Δp was modulated in mitochondria isolated from rats younger than 5 weeks. In contrast, over this age, two different flow-force relationships were obtained, i.e., uncoupler decreased Δp more than ADP + P_i at an equivalent respiratory rate.

Since the effects of almitrine have been observed in liver mitochondria isolated from rats older than 10 weeks, we reinvestigated almitrine action versus rat age. Almitrine had no effect on ATPase/ATPsynthase complex when liver mitochondria were isolated from rats younger than 5 weeks. Moreover, the transition between zero and maximal effect occurring between 5 and 9 weeks, is identical to that previously observed for the age-dependent effect of CCCP [4]. Such changes seem related to general membrane properties as suggested by lipid composition analysis.

Correspondence to: M. Rigoulet, Institut de Biochimie cellulaire et Neurochimie du CNRS, Université de Bordeaux II, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cédex, France.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; ΔpH, transmembrane difference of pH; $\Delta\Psi$, transmembrane difference of electrical potential; Δp, protonmotive force; ARLM, adult rat liver mitochondria; YRLM, young rat liver mitochondria; 18:2, linoleic acid; 22:6, docosahexaenoic acid; F.A., fatty acid.

Materials and Methods

Preparation of mitochondria

Male Wistar rats were used. Except when indicated in the legends to figures, all the experiments were carried out on 5-week-old (about 130 g body weight) 'young' or 10-week-old (about 300 g body weight) 'adult' animals.

Rats were killed by cervical dislocation and their liver was rapidly put into an ice-cold medium containing 225 mM sucrose, 1 mM EGTA and 10 mM Tris-HCl (pH 7.2). Mitochondria were isolated in the same medium according to Ref. 5. Protein concentration was estimated by the biuret method using serum albumin as a standard.

ATP / O measurement

ATP/O ratio stoichiometries were determined from the average of phosphorylation rates vs. respiratory rates at 28°C. Oxygen consumption rate was measured polarographically by using a Clark electrode. ATP production and oxygen consumption were measured in isolation medium supplied by 1 mM malate, 5 μM rotenone, 10 mM Tris-P_i and 10 mM succinate in the presence of non-limiting amounts of hexokinase, 1 mM $MgCl_2$ and 10 mM glucose. ATP production was monitored by glucose 6-phosphate formation assayed by a standard enzymatic method [6] in the protein-free neutralized extract.

Determination of ATPase activity

Mitochondria (3 mg protein) were incubated in 3 ml of medium containing 125 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.2), 5 μM rotenone and 0.25 mM valinomycin. The reaction was started by addition of 1 mM ATP. The P_i amount was measured according to Ref. 7. The oligomycin-sensitive ATPase was the difference between the rates of ATP hydrolysis observed in the absence or in the presence of 10 μg/ml oligomycin.

Lipid determination

Lipids were extracted into chloroform/methanol (2/1; v/v) by the method of Bligh and Dyer [8]. The pellet was extracted once more by 10 volumes of chloroform/methanol (2/1; v/v). The two chloroform phases were filtered on glass-wool, dried under vacuum and washed by the method of Folch et al. [9], and the crude lipid extract was stored in chloroform at −20°C until analysis.

Lipid phosphorus was determined by Macheboeuf and Delsal's method [10]. Cholesterol was measured by an enzymatic method [11].

Fatty acid composition was determined from 1 ml of the lipid extract after transformation into isopropylic esters [12]. Fatty acids were separated in a Carlo-Erba 6000 chromatograph equipped with a 25 m capillary column (0.32 mm internal diameter; CARBOWAX). Column conditions were 180°C for 5 min, rising then by 7.5°C/min to 220°C for 35 min. The injector was at 60°C and the flame ionization detector at 250°C. Helium was used as a carrier gas (flow rate 2 ml/min). Peak identifications made by comparison with reference fatty acids (Sigma) and peak areas were measured with an automatic integrator DP 700 Carlo-Erba. Quantification of each fatty acid was expressed as a percentage of the total extract.

Almitrine (see Ref. 2 for chemical structure) was a gift from Servier Laboratory, France. Before use, almitrine (powder) was solubilized in water to a final concentration of 2 mM. This solution was stored at 4°C without pH adjustment.

Results

Age-dependent changes in the almitrine effect

Fig. 1A compares the effect of almitrine on the ATPase activity of mitochondria isolated from young (YRLM) and adult rat liver (ARLM). On ARLM, almitrine inhibited oligomycin-sensitive ATPase induced by valinomycin addition, with half-inhibition at

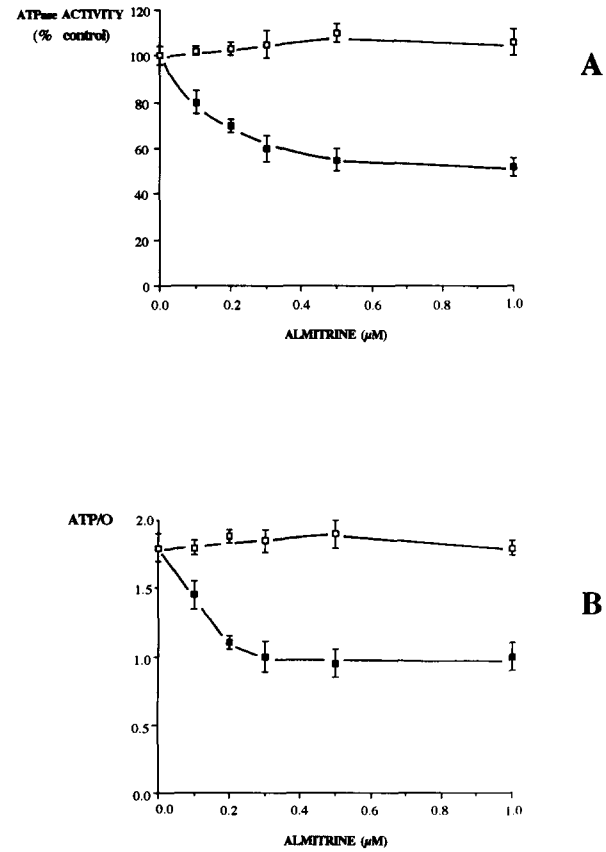

Fig. 1. Effect of almitrine on oligomycin-sensitive ATPase (A) and on ATP/O ratio (B) in YRLM (□) and in ARLM (■). Experimental procedure is described in Materials and Methods.

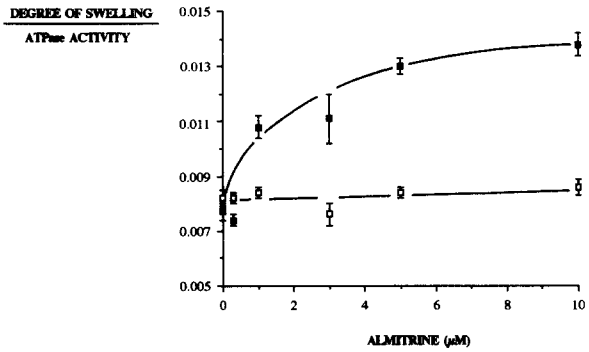

Fig. 2. Effect of almitrine on efficiency of oligomycin-sensitive ATPase of YRLM (□) and ARLM (■). The efficiency of oligomycin-sensitive ATPase was estimated by the ratio of the degree of swelling on oligomycin-sensitive activity and expressed as absorbance unit/nmol ATP per min per mg protein. Swelling was monitored at 546 nm by using an Eppendorf photometer. Mitochondria (3 mg protein) were incubated in 3 ml of the preparation medium (see Materials and Methods) supplemented with 2 mM $MgCl_2$, 2 mM ATP, 10 mM potassium acetate and 5 μM rotenone. Swelling was initiated by 0.01 μg valinomycin per ml. ATPase activity was measured when swelling was maximum and stable.

0.13 μM. Inhibition was never higher than 45%. These results are in agreement with Ref. 2 in which the ATPase activity was induced by CCCP addition. In contrast, on YRLM, almitrine did not inhibit oligomycin-sensitive ATPase activity.

Almitrine had no effect on state 3 respiration on these two kinds of mitochondria (data not shown). However, as previously shown [2], this drug induced a large decrease in the ATP synthesis rate on ARLM, and consequently a drop in the ATP/O ratio (Fig. 1B). The maximal effect was observed at 0.5 μM almitrine; under these conditions Δp was not modified (not shown but see Ref. 2). On YRLM, almitrine did not modify the ATP/O ratio (Fig. 1B).

Since the decrease in both the ATPase activity and the ATP/O ratio observed in ARLM could be the consequence of a change in ATPase/ATPsynthase

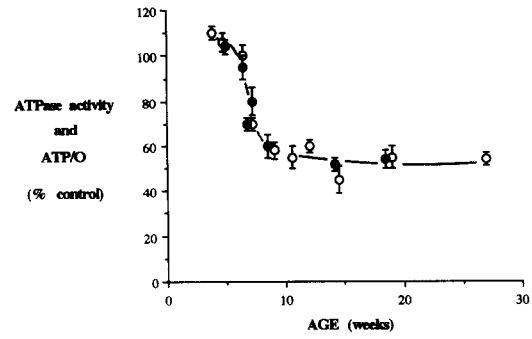

Fig. 3. Age-dependent change in almitrine effect on both ATPase activity (○) and ATP/O ratio (●). Oligomycin-sensitive ATPase activity and ATP/O ratio were obtained as indicated in Materials and Methods in the absence and presence of 0.5 μM of almitrine.

stoichiometry, we tested in YRLM the action of almitrine on the efficiency of this proton pump. A very simple system is energy-dependent potassium salt uptake, which can be followed by valinomycin-induced swelling [13–15]. The H^+-efflux catalyzed by a proton pump sustains a $\Delta\Psi$-dependent potassium and a ΔpH-dependent acetate accumulation. Therefore, energy supply (ATP) is converted into salt accumulation. We have shown, in beef heart mitochondria, that almitrine largely increases the energy-linked swelling supported by oligomycin-sensitive ATPase, although this activity is slightly inhibited. Thus, the ratio between the degree of swelling, reflecting the size of the transmembrane salt gradient, and oligomycin-sensitive ATPase activity, can be regarded as a measure of ATPase efficiency. As expected, Fig. 2 shows that this ratio was largely increased by almitrine in the case of ARLM. In contrast, it remained constant in YRLM whatever the almitrine concentration. Therefore, not all the effects of almitrine observed in ARLM were present in YRLM.

The percentage of the ATPase activity and ATP/O ratio remaining after almitrine addition (0.5 μM) was

TABLE I

Cholesterol and lipid phosphorus contents and fatty acid composition of rat liver mitochondria isolated from young and adult rats

Liver mitochondria were isolated from young (5.5 ± 0.5 weeks) and adult rats (14.4 ± 4 weeks) and lipids were extracted as described in Materials and Methods. Data are presented as means ± S.D. of 8 experiments. Statistical significance was determined by Student's *t*-test: [a] $P < 0.01$; [b] $P < 0.001$.

Liver mitochondria isolated from:	Young rats	Adult rats
Cholesterol (nmol/mg protein)	24.1 ± 2.1	25.8 ± 2.9
Lipid phosphorus (nmol/mg protein)	304 ± 20	263 ± 26
% Total fatty acids		
14:0	0.20 ± 0.05	0.19 ± 0.03
15:0	0.43 ± 0.20	0.39 ± 0.06
16:0	18.1 ± 0.80	17.1 ± 0.80
16:1	0.60 ± 0.22	0.58 ± 0.33
17:0	1.21 ± 0.35	0.85 ± 0.10
18:0	20.4 ± 2.20	18.8 ± 2.20
18:1	6.40 ± 1.60	7.60 ± 1.00
18:2	19.9 ± 1.00	23.1 ± 0.80 [b]
18:3	0.34 ± 0.06	0.30 ± 0.06
20:2	0.57 ± 0.18	0.68 ± 0.20
20:3	0.84 ± 0.11	1.06 ± 0.13
20:4	19.2 ± 0.90	19.9 ± 1.10
20:5	0.60 ± 0.21	0.70 ± 0.16
22:5	1.01 ± 0.21	0.80 ± 0.19
22:6	9.60 ± 0.40	7.26 ± 0.30 [b]
Polyunsaturated fatty acids	52.3 ± 0.80	54.0 ± 0.50 [a]

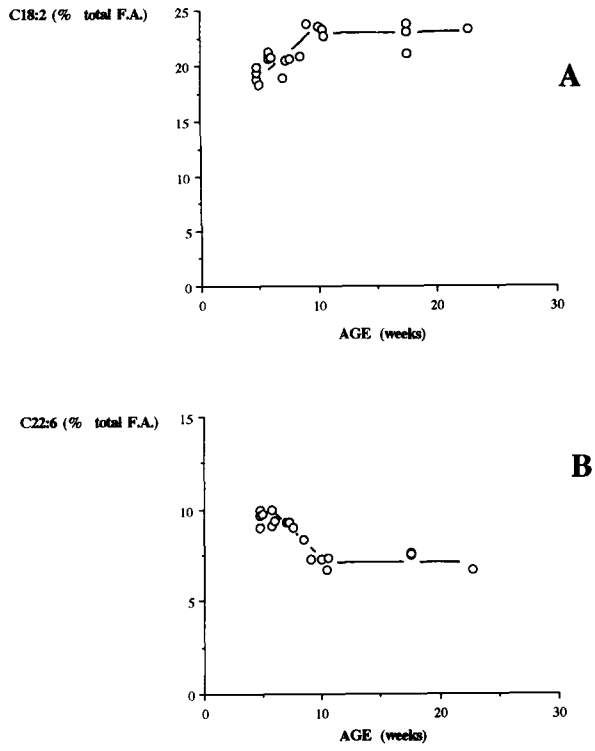

Fig. 4. Age-dependent change in linoleic acid (A) and docosahexaenoic acid (B) content of rat liver mitochondrial membrane. Fatty acids were separated by gas chromatography as described in Materials and Methods. Quantification of each fatty acid was expressed as the percentage of the total extract.

measured versus rat age. Fig. 3 shows that there were the same relationships between both the ATP/O ratio and ATPase activity, and the age of the animal. A transition was observed when the rat was about 6–9 weeks old: under this age there was no inhibitory effect of almitrine; over 11 weeks, the effect of almitrine was maximal and remained constant.

Age-dependent changes in the polyunsaturated fatty acid content

First, we compared the membrane lipid composition of YRLM and ARLM. As shown in Table I, cholesterol and phospholipid contents were not significantly changed. Moreover, it should be noted that the phospholipid composition also remained unchanged (not shown). Analysis of the fatty acid distribution showed that only the linoleic (18:2) and docosahexaenoic (22:6) acids were significantly increased and decreased, respectively. Moreover, the percentage of polyunsaturated fatty acids was slightly increased (see Table I).

The percentage of linoleic (Fig. 4A) and docosahexaenoic (Fig. 4B) acids were determined versus rat age. In both cases, the changes occurred between 5 and 10 weeks. After that the two fatty acid contents was not significantly modified at least until 22 weeks. Moreover, the transitions observed in Fig. 3 concerning the almitrine effect on ATPase/ATPsynthase, and in Fig. 4 concerning both membrane 18:2 and 22:6 contents, present the same age-dependence, even though the latter are more gradual.

Discussion

As reported in this work, the effects of almitrine, previously described in yeast and beef heart mitochondria [1–3], are also observed in liver mitochondria isolated from rats older than 6–7 weeks. However, for rats younger than 5 weeks, almitrine has no effect on the ATPase/ATPsynthase complex. It was worth noting that the decrease of both ATPase activity and the ATP/O ratio as a function of almitrine concentration in ARLM are strictly related. Moreover, the ATPase activity and ATP/O ratio, measured in the presence of 0.5 μM almitrine, change in the same manner versus animal age. Given these facts, it is likely that both phenomena are closely linked. This is in favour of our previous interpretation of the inhibitory effect of almitrine on the ATPase/ATPsynthase complex as being a direct consequence of an H^+/ATP stoichiometry increase [2].

An identical age-dependent effect of CCCP on oxidative phosphorylation in rat liver mitochondria has been reported [3,4]. In YRLM, CCCP acts as a classical protonophoric uncoupler by increasing the H^+-leak, but in ARLM, CCCP induces a decrease in the proton pumping efficiency of the respiratory chain in addition to this effect (see also Ref. 16). Although the transition occurs strictly at the same age, it is important to note that the effects of almitrine and CCCP are very different: almitrine increases the mechanistic stoichiometry of the ATPase/ATPsynthase, and CCCP induces a slipping in the respiratory chain. Indeed, the fact that different mechanisms of change in stoichiometry concerning different compounds appear at the same period of life, seems to indicate that pump sensitivity to a given drug is related to general membrane properties.

The membrane lipid composition of rat liver mitochondria is extensively described (see Ref. 17 for review). In aging-dependent studies, the authors observe an increase in cholesterol content [18–21] and a decrease in lipid phosphorus content [20,21]. More generally, various diseases lead to large modifications in the polyunsaturated fatty acid pattern: particularly arachidonic and docosahexaenoic acids undergo degradation to form lipid hydroperoxides [22,23]. Such changes in the membrane lipid content and/or in the lipid composition alter lipid-protein interactions which are, at least in part, responsible for the modifications in membranal protein activities.

In our study, we observed neither changes in cholesterol or lipid phosphorus contents, nor a decrease in total polyunsaturated fatty acids. Moreover, it should be noted that no significant difference was observed in any of the oxidative phosphorylation activities between YRLM and ARLM in control experiments in the absence of almitrine (not shown but see Ref. 4). However, although we noted only a few modifications in polyunsaturated fatty acid distribution, i.e., an increase in linoleic acid (16%) and a decrease in docosahexaenoic acid (24%) (Table I), they could play a key role regarding the sensitivity of the mitochondrial proton pump to some drugs. Indeed, both these variations and those of the almitrine and CCCP effects appear at the same age. This suggests that the action of these drugs on membranal pumps is strongly dependent on the lipidic environment, although the slope of the drug transitions is steeper.

In conclusion, we underline the fact that, in rat liver mitochondria, the possibility of a change in proton pump stoichiometry induced by some drugs appears from around 7 weeks. Biochemical studies are usually carried out on liver mitochondria isolated from rats weighing 200 to 250 g. For the Wistar bride used in our laboratory, the animals with such a body weight are about 7 to 9 weeks old, which is the transition period during which the almitrine effect appears. This could be one of the sources of contradictory results in studies concerning the effect of various drugs on oxidative phosphorylation.

Acknowledgements

This work was generously supported by I.R.I.S. (Institut de la Recherche Internationale Servier) and by grants from the Conseil Régional d'Aquitaine. We thank Dr. Ray Cooke for his linguistic assistance.

References

1 Rigoulet, M., Ouhabi, R., Leverve, X., Putod-Paramelle, F. and Guérin B. (1989) Biochim. Biophys. Acta 975, 325–329.
2 Rigoulet, M., Fraisse, L., Ouhabi, R., Guérin, B., Fontaine, E. and Leverve, X. (1990) Biochim. Biophys. Acta 1018, 91–97.
3 Rigoulet, M. (1990) Biochim. Biophys. Acta 1018, 185–189.
4 Jumelle-Laclau, M., Rigoulet, M. and Guérin B. (1992) in Biothermokinetics (Westerhoff, H.V., ed.), Intercept, Andover, in press.
5 Klingenberg, M. and Slencka, W. (1959) Biochem. Z. 331, 486–495.
6 Bergmeyer, H.U. (Ed.) (1970) Methoden der enzymatishen Analyse, 2nd Edn., Verlag Chemie, Weinheim.
7 Summer, J.B. (1944) Science 100, 413–418.
8 Bligh, E.C. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
9 Folch, J.M., Lees, M. and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509.
10 Macheboeuf, M. and Delsal, J. (1943) Bull. Soc. Chim. Biol. 25, 116–120.
11 Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) Clin.Chem. 20, 470–475.
12 Peuchant, E., Wolff, R.G., Salles, C. and Jensen R. (1989) Anal. Biochem. 181, 341–349.
13 Chappel, J.B. and Crofts, A.R. (1965) Biochem. J. 95, 393–402.
14 Pressman, B.C. (1965) Proc. Natl. Acad. Sci. USA 53, 1076–1083.
15 Chappell, J.B. and Crofts, A.R. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., eds.), pp. 293–314, Elsevier, Amsterdam.
16 Luvisetto, S., Pietrobon, D. and Azzone, G.F. (1987) Biochemistry 26, 7332–7338.
17 Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42.
18 Grinna, L.S. (1977) Mech. Ageing Dev. 6, 197–205.
19 Horton, A.A. and Spencer, J.A. (1981) FEBS Lett. 133, 139–141.
20 Vorbeck, M.L., Martin, A.P., Long, J.W. Jr., Smith, J.M. and Orr, R.R., Jr. (1982) Arch. Biochem. Biophys. 217, 351–361.
21 Paradies, G. and Ruggiero, M. (1991) Arch. Biochem. Biophys. 284, 332–337.
22 Vladimirov, Y.A., Olenov, V.I., Suslova, T.B. and Cheremisina, Z.P. (1980) in Advances in lipid Research (Paoletti, R. and Kritchevsky, D., eds.), pp 173–249, Academic Press, New York.
23 Sevanian, A. and Hochstein, P. (1985) Annu. Rev. Nutr. 5, 365–390.