Quebec Cooperative Study
of Friedreich's Ataxia

Friedreich's Ataxia in Northern Italy
II. Biochemical Studies in Cultured Cells

B. BERTAGNOLIO, G. UZIEL, E. BOTTACHI, G. CRENNA, A. D'ANGELO, S. DI DONATO

SUMMARY: *Pyruvate and palmitate oxidations by cultured fibroblasts suspensions were measured in optimized conditions and proved to be within normal range in the cells from Friedreich's patients. However, when pyruvate oxidation was measured by direct assay of the pyruvate dehydrogenase complex, this enzyme activity proved to be significantly lower in Friedreich's than in controls' cells. These abnormalities were not observed when the cells were sonicated. Moreover, lipoamide dehydrogenase activity, Km and Vmax were within the normal range in Friedreich's cells. These data suggest that the low activities of the PDH complex are not a primary defect in Friedreich's ataxia, but are more likely related to membrane abnormalities in Friedreich's cells.*

RÉSUMÉ: *Nous avons mesuré dans des conditions optimales l'oxidation par des suspensions de fibroblastes en culture du pyruvate et du palmitate. Celles-ci s'avérèrent normales dans les cellules de patients souffrant d'ataxie de Friedreich. Cependant lorsque l'oxidation du pyruvate fut mesurée par un dosage direct du complexe de la pyruvate dehydrogenase, l'activité de cet enzyme s'avéra significativement diminuée chez les Friedreich par rapport aux témoins. Ces anomalies ne furent pas observées après sonication des cellules. L'activité, le Km et le Vmax de la lipoamide dehydrogenase furent trouvés dans les limites normales. Ces données suggèrent que les basses activités du complexe PDH ne sont pas un défaut primaire dans l'ataxie de Friedreich, mais reflètent plutôt une anomalie membranaire des cellules de Friedreich.*

From Instituto Neurologico "C. Besta", Milano, Italy.

Reprint requests for the complete supplement on Friedreich's Ataxia (phase three) to: Dr. André Barbeau, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada, H2W 1R7.

INTRODUCTION

The primary metabolic defect in Friedreich's ataxia (F.A.) has not been identified. However, data reported by Blass et al. (1976) suggested that the activities of the pyruvate-dehydrogenase and α-KGDHC) were low in glycerol-homogenates of cultured fibroblasts from these patients. Since these two enzymes complexes have a common component, PDH-E_3 or lipoamide-dehydrogenase (LAD), this activity was investigated by Kark and collaborators, (1978, 1979) and found to be abnormal in the cell homogenates of some Friedreich's patients. These authors showed that PDHC and α-KGDHC activities in platelet preparations from patients with spinocerebellar degenerations were lower than in control platelets. These data were not confirmed by Barbeau et al. (1976), Melançon et al. (1979) and by Stumpf and Parks (1978, 1979). The latter authors reported normal activities of PDH, α-KGDH and LAD in sonicated fibroblasts cultured from Friedreich's patients: at variance with the data of Rodriguez-Budelli et al., (1978) the kinetics of LAD was comparable in the controls' and the patients' cells. Moreover, Stumpf and Parks (1979) showed that oxidation rates by whole cell suspensions of many substrates, including pyruvate and palmitate, from F.A. patients tallied with the rates for control cells.

In our group of F.A. patients, diagnosed according to strict clinical criteria (see D'Angelo et al. this issue), we measured the oxidation rates of pyruvate and palmitate in whole cell suspensions in optimized conditions, as previous experiments in our laboratory had shown that in trypsin-suspended cells, cofactors necessary for maximal oxidation rates of these

https://doi.org/10.1017/S0317167100022964 Published online by Cambridge University Press

substrates were lacking. Given the discrepancies between the results of Kark et al. (1979) and Blass et al. (1976) and those of Stumpf and Parks (1979) concerning the PDH activity in F.A. cells, we measured the activities of PDHC and α-KGDHC and LAD by different methods of cell disruption in order to establish whether the contradictory results were the outcome of different methods for solubilizing the PDH and α-KGDH complexes. Because of the putative role of carnitine-acetyl-transferase (CAT) in the control of pyruvate oxidation (Di Donato et al., 1979) this activity was determined in cultured cells, together with the activity of NADPH dependent glutamate-dehydrogenase (GDH) an enzyme found abnormal in cerebellar degeneration of the olivopontocerebellar type (Plaitakis et al., 1979).

MATERIALS AND METHODS

Cultures of human fibroblasts from 26 normal and diseased controls (10 normal, 2 neuronal-ceroid-lipofucsinosis, 2 acid maltase deficiency, 2 acid maltase deficiency heterozygotes, 2 metachromatic leukodystrophy, 2 metachromatic leukodystrophy heterozygotes, 1 polymyositis, 1 mucolipidosis III, 2 Tay-Sachs disease, 2 Tay-Sachs heterozygotes and 2 undiagnosed progressive encephalopathies), 9 ataxic patients not affected by F.A. (4 olivopontocerebellar atrophy, 1 Holmes cerebellar degeneration, 2 transient ataxias with normal PDH activity, 1 α-neuraminidase deficiency, 1 dominant CMT disease) and 11 patients with F.A. (patients 1 to 9 and 12-13 of table 1 of our previous paper), were grown from skin biopsies, in Eagle MEM supplemented with 10% foetal calf serum, buffered at pH 7.4 with Na bicarbonate in 75 cm^2 Falcon Flasks as previously described (Di Donato et al., 1977). Fibroblasts, subcultured 5 to 15 times, were harvested at 10 min incubation at 37° with 0.25% trypsin in Tris-buffered saline (TBS) solution, containing 1.0 mM EDTA, pH 7.4. Cells were pelletted by centrifugation for 5 min at 2600 rpm and washed twice with TBS. Pyruvate 1-^{14}C and pyruvate 2-^{14}C oxidations were measured in TBS suspended cells in 20 ml scintillation vials, stoppered with rubber caps, with a strip of paper moistened with 50 μl of 1 M Hyamine Hydroxide in methanol. Before the addition of labelled pyruvate, the cells were preincubated 15' in TBS with 1.0 mM L-aspartate in order to maximize pyruvate oxidation. Incubations were then performed in a oscillating bath (25 strokes/min) at 37° C (30 min for pyruvate-1-^{14}C, 0.1 mM final concentration; 60 min for pyruvate-2-^{14}C, 0.1 mM final concentration). The radioactive CO_2, formed during incubation times, and trapped by Hyamine Hydroxide was counted in 10 ml of InstaGel (Packard) in a Tricarb scintillation counter. Blanks were obtained by killing the cells with methanol. Palmitate-1-^{14}C oxidation was measured by the same method, except that incubation mixtures contained 0.1 mM albumin-bound palmitate-1-^{14}C (albumin/palmitate ratio 0.14) and that cells were preincubated 15 minutes with 0.5 mM L-carnitine before adding palmitate in order to maximize palmitate oxidation. PDHC and α-KGDHC were measured in cell homogenates by the current radiochemical methods (Blass et al., 1976) while LAD activity was measured by the current spectrophotometric method (Di Donato et al., 1979; Stumpf and Parks, 1978): kinetics of LAD versus lipoamide as substrate were evaluated. For the LAD assay the cells were sonicated (Rodriguez-Budelli and Kark, 1978; Stumpf and Parks, 1978). On the other hand, for PDHC and α-KGDHC two different methods of cell disruption were used (i.e. homogenization in glycerol and sonication) in order to compare the effect of homogenation on the activities of the two enzymes. The activity of carnitine-acetyltransferase (CAT) was measured in cells sonicated by the radiochemical method previously described (Di Donato et al., 1979). Protein was measured with the method of Lowry et al., (1951). NADPH dependent glutamate-dehydrogenase was measured in fibroblast sonicated by the method of Schmidt (1974).

RESULTS

Preliminary experiments performed in our laboratory showed that a) suspensions of cultured fibroblasts from F.A. strains oxidized pyruvate-1-^{14}C, pyruvate-2-^{14}C and palmitate-1-^{14}C at the same rates as control strains; b) preincubation of normal cells with 1 mM l-aspartate increased pyruvate oxidation with both pyruvate-1-^{14}C and pyruvate-2-^{14}C (mean increase in pyruvate-1-^{14}C oxidation was 146 ± 24% SD; for pyruvate-2-^{14}C it was 124 ± 15% in 20 normal human strains); c) preincubation of cells with 0.5 mM l-carnitine palmitate-1-^{14}C oxidation (means increase 196 ± 49 S.D. in 18 human strains). Oxidation rates for pyruvate and palmitate in F.A. and controls' cells in the presence of these cofactors are shown in table 1. No difference between the oxidation rates for pyruvate and palmitate between controls and F.A. fibroblasts was evidenced: moreover, the mean in-

TABLE 1

*Pyruvate and Palmitate Oxidations by Whole Cell Suspensions from Controls' and Friedreich's Fibroblasts**

	Controls	Friedreich's
Pyruvate 1-^{14}C***	380 ± 118 (12) (222 - 598)	400 ± 139 (8) (204 - 590)
Pyruvate 2-^{14}C**	149 ± 53 (12) (58 - 217)	132 ± 30 (8) (88 - 163)
Palmitate 1-^{14}C***	30 ± 12 (18) (16.5 - 52.3)	31.0 ± 5.0 (10) (21.9 - 41.0)

*Values are expressed as mean picomoles CO_2-^{14}C formed/min/mg cell protein ± SD
**Preincubated with 1.0 mM L-aspartate
***Preincubated with 0.5 mM L-carnitine
The range of activity and the number of cell lines analyzed are indicated in brackets.

crease in oxidation rates for pyruvate and palmitate due to L-aspartate and L-carnitine were comparable in controls and in F.A. patients (data not shown). The activities of PDHC and α-KGDHC in controls, ataxic and F.A. patients are given in table 2. The activities of PDHC and α-KGDHC measured in glycerol-homogenates, did not significantly differ in controls and ataxic patients' whereas PDHC was significantly lower in the group of F.A. patients than in the controls ($P < 0.001$) and ataxic patients ($P < 0.02$). α-KGDH activity was lower in F.A. than in controls' cells but the difference was not statistically significant ($P < 0.05$). From table 2, however, it is clear that when PDHC and α-KGDHC were measured in sonicated cells, the activities of both enzyme complexes were lower than in glycerol homogenates and no statistically significant difference could be found between F.A. patient and controls. Moreover, specific activity, Km with lipoamide as substrate and Vmax of LAD did not significantly differ in controls and F.A. cells (table 3). No differences between the activities of CAT and GDH in F.A. and control cells were observed. NADPH-dependent GDH activity however, was significantly lower, than in controls, in the cultured cells from 5 patients with olivopontocerebellar atrophy (data not shown).

TABLE 2

Activity of PDHC and α-KGDHC in Cultured Fibroblasts from Controls Ataxic and Friedreich's Patients

Enzymes in Glycerol Homogenates	**Control**	**Ataxic**	**F.A.**
PDHC*	569 ± 115 (20) (385 - 764)	544 ± 130 (9) (420 - 819)	404 ± 109 (11) (322 - 624)
α-KGDHC*	667 ± 173 (18) (389 - 1168)	651 ± 152 (9) (474 - 965)	521 ± 227 (9) (313 - 926)
Enzymes in Cell Sonicates			
PDHC*	216 ± 36 (73 - 323)	ND	248 ± 80 (6) (121 - 423)
α-KGDHC*	357 ± 36 (6) (147 - 535)	ND	367 ± 60 (6) (177 - 496)

*Activities are expressed as mean picomoles/min/mg cell protein ± S.D.
**$P < 0.001$ versus controls and $P < 0.02$ versus ataxic.
ND = Not determined.
Each value for controls and patients is the mean of two duplicate determinations.
The range of activity and the number of cell strains are indicated in brackets.

TABLE 3

Activities of Lipoamide Dehydrogenase, Carnitine — Acetyl Transferase and Glutamate Dehydrogenase in Cultured Cells from Controls and F.A. Patients*

	Controls	**F.A.**
LAD	77 ± 19 (15) (44 - 116)	66 ± 9 (5) (57 - 77)
LAD km (mM lipoamide)	1.80 ± 0.19 (15) (1.4 - 2.6)	1.84 ± 0.47 (7) (1.3 - 2.6)
LAD Vmax	102 ± 38 (13) (61 - 208)	94 ± 10 (7) (83 - 107)
CAT	0.33 ± 0.03 (16) (0.25 - 0.40)	0.30 ± 0.05 (6) (0.25 - 0.37)
GDH	65.4 ± 8.7 (10) (53.3 - 82.5)	62.6 ± 11.0 (7) (46.1 - 83.6)

*Activities for the 3 enzymes are expressed as mean nanomoles/min/mg cell protein ± S.D. each value for controls and F.A. cells in the mean of two duplicate determinations.

COMMENT

The results of preliminary experiments in our laboratory demonstrated that suspensions of cultured cells from F.A. patients oxidized pyruvate and palmitate at the same rate as controls. As cell suspensions, in the absence of added L-aspartate and L-carnitine, oxidized pyruvate and palmitate at a low rate, we measured these activities in the presence of adequate amounts of these cofactors: again no difference in the oxidation rates of pyruvate and palmitate could be detected between F.A. and controls cells (table 1). Therefore, our data in whole cells suspensions, obtained using both pyruvate-1-^{14}C and pyruvate-2-^{14}C, did not suggest defects in pyruvate oxidations in cultured F.A. cells, in agreement with the data of Barbeau et al., (1976) and Stumpf and Parks (1978). The use of both substrates ruled out defects of pyruvate decarboxylation and defects of pyruvate-derived acetyl-CoA utilization in the Krebs cycle. However when the activity of the PDH complex was measured by direct enzyme assay by the glycerol-homogenate method of Blass et al. (1976) a significant reduction of the PDHC activity was observed in F.A. cells versus control cells and versus ataxic non-Friedreich patients, ($P < 0.001$ and $P < 0.02$ respectively). These data are in agreement with the data reported by Blass and coworkers (1976) and differ from those reported by Stumpf and Parks (1978): the activity of α-KGDHC was not statistically different in F.A. cells

https://doi.org/10.1017/S0317167100022964 Published online by Cambridge University Press

from that of the controls ($P < 0.05$) and ataxic patients', in our patients as well as those reported by Blass et al., (1976).

However, when sonicated cells were used to measure PDHC and α-KGDHC, as in the assay suggested by Stumpf and Parks, 1978, the activities of both the controls and the Friedreich'sf cells were lower than those obtained in glycerol-homogenates and no statistically significant difference could be found between Friedreich's patients and controls (Table 2), in agreement with Stumpf and Parks (1978). Therefore, the discrepancies between the data reported by the authors on the PDHC activity could be tentatively attributed to differences in the assay methods. Our data in table 3 show that the low activity of the PDHC observed in F.A. glycerol-homogenates was not a consequence of the low activity of the third component of the PDH complex. LAD specific activity in F.A. cells was normal as well as its Km (with lipoamide and substrate) and Vmax. Therefore, low activity in F.A. cells of the PDH complex was not correlated to: a) abnormalities of pyruvate oxidation by whole cells as the oxidations rates of both pyruvate-1-^{14}C and pyruvate-2-^{14}C were normal in F.A. fibroblasts in the presence and in the absence of L-aspartate; b) abnormalities of the lipoamide dehydrogenase. Moreover, low activities of the PDH complex were found in Friedreich's fibroblasts glycerol-homogenates but not in sonicated cells (table 2). These findings suggest that the low activity of the PDH complex is not a primary defect in F.A. but rather that it may perhaps be related to a) subtle abnormalities of the enzyme complex structure expressed in vitro only when conservative homogenization methods are used; b) abnormal membrane composition of the F.A. cells, as recently suggested by Barbeau (1979) resulting in different sensitivities of the cells to the disruption procedures. Work is in progress to test the latter hypothesis. The fact that CAT and GDH activities, found abnormal in some ataxic patients (Di Donato et al., 1979; Plaitakis et al., 1979) were in the normal range (table 3) in F.A. cells, suggested that these abnormalities are restricted to ataxic conditions other than F.A., namely olivopontocerebellar atrophy for GDH (Plaitakis et al., 1979). The determination of these enzymes in cultured cells, if proven reliable and significant in clinically defined diseases, may be a useful tool for discriminating ataxic phenotypes close to F.A. from the Friedreich patients.

EDITOR'S NOTE

The above paper is the same text which has been accepted for publication in the Italian Journal of Neurological Sciences (September, 1980) and is reproduced here with the express permission of the IJNS editor and the authors, because of its pertinence to the Quebec Cooperative Study on Friedreich's Ataxia and in acknowledgement of international cooperation on this important subject.

REFERENCES

BARBEAU, A., BUTTERWORTH, R.F. NGO, T., BRETON, G., MELANÇON, S., SHAPCOTT, D., GEOFFROY, G. and LEMIEUX, B. (1976). Pyruvate metabolism in Friedreich's ataxia. Can. J. Neurol. Sci., 3: 379-388.

BARBEAU, A. (1979). Friedreich's ataxia: an overview. Can. J. Neurol. Sci., 6: 311-319.

BLASS, J.P., KARK, R.A.P. and MEMON, R.K. (1976). Low activities of the pyruvate-dehydrogenase and oxoglutarate dehydrogenase complexes in five patients with Friedreich's ataxia. New Engl. J. Med., 295: 62-67.

D'ANGELO, A., DI DONATO, S., CRENNA, G., NEGRI S., BEULCHE, F., UZIEL, G. and BOERI, R. (1980). Friedreich ataxia I. Clinical, neurophysiological and biochemical studies in vivo. This issue.

DI DONATO, S., WIESMANN, U.N. and HERSCHKOWITZ, N.N. (1977). Membrane adsorption and internalization of (^{14}C) chloroquine by cultured human fibroblasts. Biochemical Pharmacol., 26: 7-10.

DI DONATO, S., RIMOLDI, M., MOISE, A., BERTAGNOLIO, B. and UZIEL, G. (1979). Fatal ataxic encephalopathy and carnitine acetyltranspherase deficiency: a functional defect of pyruvate oxidation? Neurology, 29: 1578-1583.

KARK, R.A.P. and RODRIGUEZ-BUDELLI, M. (1979). Pyruvate-dehydrogenase deficiency in spinocerebellar degeneration. Neurology, 29: 126-131.

LOWRY, O.H., ROSENBROUGH, N.J., FERR, A.L. and RANDELL, R.J. (1951). Protein measurement with the Folin-phenol reagent. J. Biol. Chem., 193: 265-268.

MELANÇON, S.B., POITIER, M., DALLAIRE, L., ROLLIN, R., FONTAINE, G. and GRENIER, B. (1979). Pyruvate-dehydrogenase, lipoamide-dehydrogenase and citrate-synthase activity in fibroblasts from patients with Friedreich's and Charlevoix-Saguenay ataxias. Can. J. Neurol., Sci., 6: 241-242.

PLAITAKIS, A., NICKLES, W.J. and DESNICH, R.S. (1979). Glutamate-dehydrogenase deficiency in 3 patients with spinocerebellar ataxia: a new enzymatic defect? Annals Neurology, 6: 148 (abs)

RODRIGUEZ-BUDELLI, M. and KARK, R.A.P. (1978). Kinetic evidence for a structural abnormality of lipoamide-dehydrogenase in two patients with Friedreich's ataxia. Neurology, 28: 1283-1286.

SCHMIDT, E. (1974). Glutarate-dehydrogenase in: Bergmeyer H.U. Methods in Enzymatic Analysis Academic Press, New York, 2nd Edition, pp 650-656.

STUMPF, D.A. and PARKS, J.K. (1978). Friedreich's ataxia. I. Normal pyruvate-dehydrogenase complex activity in platelets. Annals Neurology, 4: 366-368.

STUMPF, D.A. and PARKS, J.K. (1979). Friedreich's ataxia. II. Normal kinetics of lipoamide-dehydrogenase. Neurology, 29: 820-826.