Diabetologia 2, 346–352 (1966)

Respiration and Phosphorylation of Mitochondria Isolated from the Skeletal Muscle of Diabetic and Normal Subjects*

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Received August 5, 1966

Summary. A group of diabetics, well controlled as far as blood sugar, free fatty acids and acetoacetate were concerned but with a high frequency of diabetic angiopathy in different organs, was compared with a group of controls in terms of the respiration and phosphorylation reactions in mitochondria isolated from skeletal muscle. Two keto-acidotic patients were also studied. The reactions studied were: respiration with glutamate or pyruvate plus malate as substrates, and also ratios of esterified inorganic orthophosphate to oxygen consumed and respiratory control with these substrates. The respiratory control in the absence of adenosine triphosphate was measured for pyruvate plus malate, and also the influence of serum albumin on this. Adenosine triphosphatase was measured under basal conditions and after maximal activation with 2,4-dinitrophenol. No certain differences were found between the non-ketotic diabetics and controls. Caution must be exercised in the interpretation of certain of the results because of difficulties in standardization of the mitochondrial isolation procedure in a patient material with wide ranges of age, infiltration of fibrous material in muscles, atrophy of muscles and other factors.

Respiration et phosphorylation des mitochondries isolées du muscle du squelette de l'homme atteint de diabète sucré et de l'homme normal.

Résumé. Un groupe de diabétiques, bien contrôlés au point de vue de la glycémie, des acides gras et de l'acéto-acétate, mais fréquemment porteurs d'angiopathie diabétique dans différents organes, a été comparé à un groupe de témoins en ce qui concerne les réactions de respiration et de phosphorylation dans les mitochondries isolées du muscle du squelette. Deux patients céto-acidosiques ont été également étudiés. Les réactions étudiées étaient: la respiration en présence de glutamate ou de pyruvate plus malate en tant que substrats, et également les rapports de l'orthophosphate inorganique estérifié sur l'oxygène consommé et le contrôle respiratoire avec ces substrats. Le contrôle respiratoire en l'absence d'adénosine triphosphate était mesuré pour le pyruvate plus malate et aussi l'influence de la sérum-albumine. L'activité adénosine triphosphatasique était mesurée dans des conditions basales et après une activation maximale avec du 2,4-dinitrophénol. Aucune différence certaine n'a été trouvée entre les diabétiques non-cétosiques et les témoins. On doit interpréter avec prudence certains résultats en raison des difficultés de standardisation des procédés d'isolement des mitochondries dans des prélèvements tissulaires effectués sur des patients d'âge très différent, présentant une infiltration fibreuse des muscles, de l'atrophie des muscles, ainsi que d'autres facteurs.

Atmung und Phosphorylierung in isolierten Skelettmuskelmitochondrien beim menschlichen Diabetes.

Zusammenfassung. Bei einer Gruppe von Diabetikern, deren Stoffwechsel auf Grund der Bestimmungen des Blutzuckers, der freien Fettsäuren und der Acetessigsäure gut eingestellt war, die aber in verstärktem Maße diabetische Gefäßerkrankungen in verschiedenen Organen aufwiesen, wurde im Vergleich zu einer Kontrollgruppe die Atmung und Phosphorylierung in isolierten Mitochondrien von Skeletmuskeln gemessen. Außerdem wurden 2 keto-acidotische Patienten untersucht. Im einzelnen bestimmten wir: Die Atmung mit Pyruvat oder Glutamat plus Malat als Substrat, ferner das Verhältnis von esterifiziertem, anorganischem Orthophosphat zum Sauerstoffverbrauch und die Kontrolle der Atmung bei diesen Substraten. Der Einfluß, den Serum-Albumin auf die Atmungskontrolle ausübte, und diese selbst wurden in Abwesenheit von ATP und in Gegenwart von Pyruvat und Malat gemessen. Die Aktivität der ATPase wurde unter Ausgangsbedingungen und nach stärkster Stimulierung durch 2,4-Dinitrophenol bestimmt. Zwischen den Kontrollpersonen und nicht ketoacidotischen Diabetikern ergaben sich keine sicheren Unterschiede. Ein Teil der Ergebnisse muß allerdings besonders vorsichtig interpretiert werden, da das Isolierungsverfahren für die Mitochondrien nur schwer zu standardisieren ist, weil im Patientengut sehr starke Schwankungen in bezug auf Alter, Bindegewebseinlagerung in die Muskulatur, Muskelatrophie und andere Faktoren bestehen.

Key-words: Diabetes mellitus, skeletal muscle, mitochondria, respiration, phosphorylation, free fatty acids, ketosis, ATP: ase, angiopathy.

Cell respiration in diabetes mellitus in the experimental animal has been the subject of much research with partly conflicting results. In studies on mitochondria isolated from tissues of diabetic animals, VESTER and STADIE [26] as well as HALL et al. [13] found a deficient respiration and phosphorylation, whereas other investigators were unable to find such impairments [21], 2.

Recent research on the metabolism of plasma free fatty acid (FFA) seems to have interesting implications for mitochondrial metabolism in diabetes mellitus. It has been shown that an increase of FFA is an early

* Reported briefly at the Fifth Congress of International Diabetes Federation, Toronto, Canada, July 23, 1964.

Reported at the Meeting of the Scandinavian Association for Diabetes Mellitus Research in Helsingör, Denmark, December 12, 1964.

pronounced metabolic error in diabetes in the human [3], producing an increased outflow of FFA from plasma into tissues [12, 6]. By competitive mechanisms at mitochondrial level this probably produces a decreased pyruvate oxidation [9, 7, 10], possibly corresponding to the impaired pyruvate tolerance in diabetes mellitus in the human [20, 19]. Furthermore fatty acids added *in vitro* to isolated mitochondria cause severe derangements of mitochondrial metabolism, both as far as phosphorylation reactions [22, 15, 23] and electron transport [4] are concerned. To what extent corresponding phenomena might occur *in vivo* is unknown.

FFA concentration in plasma has been considered as a regulatory mechanism for oxygen uptake in peripheral tissues [6] and some evidence for this view has been obtained [25, 14]. The old clinical observation of hyperthermia in diabetic ketoacidosis, might in this way be caused by the extremely high levels of FFA interfering at some level of mitochondrial metabolism.

In view of the somewhat conflicting findings in the field of metabolism of mitochondria from tissues of diabetic animals (reviewed by STADIE [24]) and particularly because of the apparent lack of studies in diabetes mellitus in the human, the present study was performed. The function of mitochondria from human skeletal muscle has been studied, utilizing a recently described method for their isolation in a functionally intact state [1].

Clinical material

The material comprised 13 patients with diabetes mellitus, age 31 to 61 years (mean 48 years). The duration of their diabetes was 1 to 18 years. The controls were 11 patients, age 42 to 61 years (mean 52 years). Patients above 65 years were excluded for reasons discussed later.

All the diabetic patients required insulin treatment. They were well regulated and in a stable metabolic condition, except for two patients who were ketoacidotic when the biopsy was performed.

The first ketotic diabetic patient (case No. 10, Table 1) agreed on withdrawal of insulin during 24 hours in order to make the examination possible. He was admitted to the hospital and his condition followed closely. At time of muscle biopsy he had the values of blood glucose, FFA and acetoacetate listed in Table 2. Furthermore, his serum total CO_2 was 7 mEq/litre, and serum sodium, potassium and chlorides 132, 4.9 and 97 mEq/litre respectively.

The second ketotic patient (case No. 13, Table 1) was admitted to the hospital in a comatous condition. Owing to a series of unusually favourable circumstances, muscle biopsy could be obtained under local anaesthesia (Lidocain, 1%, ASTRA) without delay of adequate treatment of the patient. Besides the blood metabolite values listed in Table 2, the blood pH of this patient was 6.95, $pCO_2 < 10$ mm Hg, total CO_2

Table 1. Clinical data for diabetic patients

Case No. and sex	Age	Duration of diabetes (years)	Type of diabetes	Blood pressure	Retinopathy	Nephropathy	Neuropathy	Vascular manifestation	Skin-biopsy	Muscle biopsy
1 m	59	18	JD	200/100	+++	+	+	+	m.a.	N
2 m	55	13	JD	175/95	+++	+	+	+	m.a.	m.a.
3 m	53	11	JD	120/90	0	0	+	+	m.a.	m.a.
4 m	50	12	JD	130/80	+++	+	+	+	m.a.	N
5 m	50	6	JD	150/80	—	—	—	—	—	N
6 m	44	8	JD	145/95	0	0	+	+	N	N
7 m	40	16	JD	125/70	++	0	0	+	m.a.	N
8 m	33	7	JD	130/80	+	0	0	0	m.a. ?	N
9 m	31	14	JD	130/80	++	0	+	0	m.a.	N
10 m[1]	56	12	JD	180/70	+	+	+	+	m.a.	N
11 f	61	1	MD	160/100	0	0	0	0	N	N
12 f	50	1	MD	180/100	0	0	0	0	N	N
13 f[1]	40	14	JD	130/60	+	+	+	+	—	m.a.

[1] = ketoacidosis.

Abbreviations:

Diabetic Retinopathy:
\+ = microaneurysm
++ = microaneurysm + haemorrhages with or without exudation
+++ = proliferative lesions

m.a. = microangiopathy
N = normal findings
m = male
f = female

JD: Juvenile diabetes
MD: Maturity onset diabetes

3 mEq/litre, and serum sodium, potassium and chlorides 126, 6.3 and 107 mEq/litre respectively.

The occurrence of diabetic angiopathy was thoroughly investigated. Retinopathy was graded by an ophthalmologist in 3 grades that are defined in Table 1. Constant proteinuria without signs of infection was called diabetic nephropathy. Neuropathy was considered present when there were physical signs and symptoms of motor or sensory disturbances, or a pathological electromyogram. The peripheral arteries were studied with oscillometry, X-ray of the vessels of the lower extremities and skin temperature measurements.

Histological examination was performed on a skin biopsy from the lateral part of the foot and on the muscle biopsy taken for isolation of mitochondria. The material is given in detail in Table 1.

The controls had earlier had only insignificant diseases except one patient, who was operated on for kidney tuberculosis 20 years ago. They were all admitted to the surgical service —6 because of fibroadenomatosis mammae, 1 for hyperplasia mammae, 3 for carcinoma mammae and 1 because of hydrocele testis. In all these patients the histological examination of the muscle biopsy showed no abnormality.

Methods

The investigation was performed over approximately 12 months. The examinations of the diabetics were scattered between the normals over the whole period of the investigation.

All patients were informed in detail about the procedure and its motivation. They were fasting 12 hours before biopsy, and the diabetics received no insulin during this time. At 7 p.m. the day before biopsy, 7 a.m. and at time for biopsy next day, antecubital venous blood was drawn for the determination of blood glucose [17], serum FFA [5], and acetoacetate [27] concentrations. Under light anaesthesia (0.5—1 g of Evipan-Sodium/Sodium hexobarbital, Bayer/intravenously, and nitrous oxide), 5—12 g of musculus pectoralis major was removed and immediately placed in 0.15 M KCl in melting ice. The tissue was immediately brought to the laboratory, dissected free from fat and connective tissue as far as possible, blotted and weighed, cut very finely with a pair of scissors, and homogenized in an all=glass Potter-Elvehjem homogenizer in 10 parts of KCl-Tris medium [1] in melting ice. The homogenizing pestle shaft was attached to a motor. Three movements of the homogenizing pestle down and up in the homogenizer cylinder at a rotating speed of maximally 2,000 r.p.m. was used throughout, furnishing enough homogenization of the tissue. Then the fractionation procedure described by AZZONE and co-workers [1] was followed. The final mitochondrial sediment was suspended in 2 ml of 0.25 M sucrose and 0.25 ml added to the Warburg flasks and 0.15 ml to the ATP: ase incubation tubes.

Respiration was measured by the Warburg technique, utilizing flasks of about 5 ml volume. To the main compartment of each flask was added 25 μmoles K_2HPO_4 (adjusted to pH 7.5 with HCl), 30 μmoles glucose, 8 μmoles $MgCl_2$, 25 μmoles Tris (adjusted to pH 7.5 with HCl), 50 μmoles KCl and 63 μmoles sucrose. The centre well contained 0.05 ml 20% KOH and the side arm 0.1 ml 30% trichloroacetic acid. Six flasks were set up for each experiment. Except for the additions mentioned above, the first two flasks contained 10 μmoles glutamate (potassium salt, Sigma) and 0.5 μmoles ATP (disodium salt, Sigma), one with and one without 25 units hexokinase (Sigma, crystalline). The remaining four flasks contained 25 μmoles pyruvate (sodium salt, Sigma) and 0.5 μmoles malate (potassium salt, Sigma) as substrate; two of these flasks contained hexokinase; and of the two flasks containing no ATP and no hexokinase, one contained 1 mg albumin (Armour, Bovine Serum Albumin, Fraction V).

The flasks were incubated at 37.4 °C for a 5 minute preincubation period and then for 20 or 30 minutes. The incubation was terminated by tipping the trichloroacetic acid into the main compartment. The gas phase was air, the final volume 1.2 ml, and the final pH 7.5.

ATP: ase activity was measured in a system containing 125 μmoles sucrose, 5 μmoles ATP, 25 μmoles Tris and 50 μmoles KCl in a final volume of 1.2 ml. Final pH was 7.5. 2,4-dinitrophenol -(DNP) stimulated ATP: ase activity was determined at the concentration 0.8×10^{-4} M of DNP, a concentration which gives maximal activity [1]. Samples for phosphorus determination [8] were taken after 6 minutes of preincubation and after another 30 minutes of incubation at 37 °C.

In some patients the ratio inorganic orthophosphate esterified to oxygen consumed (P/O ratio) was measured, extrapolating oxygen uptake during the preincubation period.

Respiratory control was expressed as the ratio between the oxygen uptake in the presence of hexokinase and that in the absence of hexokinase.

Protein was determined according to the method of LOWRY and co-workers [18].

Results

In Table 2 are given the values of glucose, FFA and acetoacetate in the blood of the diabetic group and the controls. Although only moderately elevated, the means of glucose, FFA and acetoacetate were higher in the diabetics except for the values of FFA and acetoacetate at 7 p.m. The two ketotic patients had markedly elevated values.

In Table 3 are given the results of the measurements of mitochondrial respiration and phosphorylation reactions. On no point was there any significant difference between non-ketotic diabetics and controls.

control was in the lower range for the latter substrate, but similarly low values were found for both normals and non-ketotic diabetics. In the absence of both hexokinase and ATP the severely ketotic patient also had the highest value recorded for oxygen uptake,

Table 2. Blood glucose, serum free fatty acids and acetoacetate of diabetics and controls. (Mean ± standard deviation)

		Controls (n = 11)	Non-ketotic diabetes (n = 11)	Ketotic patient 1	Ketotic patient 2
Blood glucose, (mg/100 ml)	7 p.m.	76 ± 13	133 ± 77	212	—
	7 a.m.	89 ± 25	143 ± 65	290	—
	at biopsy	76 ± 7	193 ± 137	312	504
Serum free fatty acids (μmoles/liter)	7 p.m.	520 ± 170	474 ± 220	1170	—
	7 a.m.	625 ± 161	739 ± 111	1923	—
	at biopsy	875 ± 360	1163 ± 226	2272	2558
Blood acetoacetate, (mg/100 ml)	7 p.m.	1.0 ± 0.3	0.8 ± 0.4	1.6	—
	7 a.m.	0.9 ± 0.4	1.4 ± 0.9	29.2	—
	at biopsy	1.0 ± 0.4	2.4 ± 1.2	33.1	45.4

Table 3. *Results of mitochondrial respiration and phosphorylation. Mean ± standard deviation, with range. Oxygen uptake data given as μatoms taken up per mg mitochondrial protein per 20 minutes*

	Controls (n = 11)			Non-ketotic diabetics (n = 11)			Ketotic patient 1	Ketotic patient 2
GLUTAMATE	Mean	S. D.	Range	Mean	S. D.	Range		
Oxygen uptake, plus hexokinase	6.40	0.91	1.73—11.49	6.41	0.90	4.22—11.58	4.32	9.20
Oxygen uptake, minus hexokinase	1.44	0.89	0.28— 2.58	1.59	0.89	0.29— 2.93	1.10	2.60
Respiratory control ratio	4.4	—	1.9 —12.8	3.9	—	1.5 —14.1	3.9	3.5
P/O, plus hexokinase**	2.5	—	—	2.7	—	—	2.3	2.7
PYRUVATE+MALATE								
Oxygen uptake, plus hexokinase	4.30	1.50	2.59— 7.68	5.11	1.40	3.28— 6.67	—*	6.57
Oxygen uptake, minus hexokinase	1.90	0.71	1.22— 2.62	1.65	0.92	0.09— 3.04	—	3.96
Respiratory control ratio	2.3	—	1.6 —10.0	3.1	—	1.7 — 5.0	—	1.7
Oxygen uptake, minus hexokinase minus ATP	0.55	0.14	0 — 1.12	0.66	0.11	0 — 1.18	—	1.64
Respiratory control ratio	8.5[1]	—	5.6 — ∞	7.1[2]	—	5.0 — ∞	—	4.0
Oxygen uptake, minus hex., minus ATP, plus albumin	0.38	0.36	0 — 1.14	0.30	0.30	0 — 1.02	—	0.93
Respiratory control ratio	16.5[1]	—	4.8 — ∞	12.9[3]	—	4.3 — ∞	—	7.0
P/O, plus hexokinase**	2.2	—	—	2.3	—	—	—	2.1
Protein yield in mitochondrial sediment, mg/g muscle	0.52	0.14	0.33— 1.49	0.62	0.11	0.36— 0.97	0.42	0.68

[1] 1 patient = ∞, not included in mean.
[2] 4 patients = ∞, not included in mean.
[3] 2 patients = ∞, not included in mean.

* Pyruvate — malate samples lost.
** Determined in 3 controls and 3 non-ketotic diabetics.

The first ketotic patient showed no apparent abnormalities in his values. The second ketotic patient, who was more severely ketotic than the first, had an oxygen uptake value without hexokinase, which was in the higher range for glutamate and the highest value registered for pyruvate plus malate. Respiratory control and oxygen uptake after addition of albumin were within the range of other observations.

Different calculations of correlation were performed. There was, however, no significant correlation between on the one hand blood glucose, FFA, aceto-

acetate and on the other any of the measured mitochondrial reactions. Nor was there any correlation between duration of the diabetes or signs and symptoms of diabetic angiopathy and any of the measured reactions.

No differences of mitochondrial reactions between men and women were apparent. There was, however, probably another factor which interfered with the measured reactions. As exemplified in Table 4, the respiration of mitochondria from the atrophic muscles of this old nondiabetic woman was very low when expressed per milligram protein. The yield of mitochondrial protein was in the lower range of those values of the other patients (Table 3). The sediment in this case was of a lighter colour and not so firmly packed as the sediments usually obtained. This phenomenon did not follow age in the cases included, since some of the oldest patients had high yield of mitochondria per wet weight of muscle and some of the youngest patients low yield.

Table 4. *Results of mitochondrial respiration in the pectoralis major muscle of a 76 year old non-diabetic woman*

Glutamate	
Oxygen uptake, plus hexokinase	1.42
Oxygen uptake, minus hexokinase	0.36
Respiratory control ratio	3.9
Pyruvate + malate	
Oxygen uptake, plus hexokinase	0.16
Oxygen uptake, minus hexokinase	0.18
Respiratory control ratio	0
Oxygen uptake, minus hexokinase, minus ATP	0
Oxygen uptake, minus hexokinase, minus ATP, plus albumin	0
Protein yield in mitochondrial sediment (mg/g muscle)	0.37

Oxygen uptake data given as μatoms taken up per mg mitochondrial protein per 20 minutes.

Table 5. *Basal and 2,4 – dinitrophenol-stimulated ATP: ase activities of skeletal muscle mitochondria from diabetics and controls. Mean ± standard deviation. Values given as μmoles inorganic orthophosphate per mg mitochondrial protein per 20 minutes*

	Controls (n = 11)	Non-ketotic diabetics (n = 11)
Basal ATP: ase	1.96 ± 0.78	1.29 ± 0.43
2,4-DNP-stim. ATP: ase	3.25 ± 1.22	3.21 ± 0.95

ATP:ase activity under basal conditions and after stimulation with DNP is given in Table 5. Here also no significant differences could be found between non-ketotic diabetics and controls.

Some of the patients, with or without diabetes, who had high FFA also had high values of basal ATP:ase. It was only possible to inhibit partially this ATP:ase activity with oligomycin (Sigma, 2 μg/ml). No correlation between yield of protein in the mitochondrial sediment and FFA was found.

Discussion

AZZONE and associates [1] recently demonstrated that the mitochondria of human skeletal muscle can be isolated in a functional state where a tight coupling of phosphorylation to oxidation is present. This was confirmed in the present work, where the same techniques were utilized.

Since it was planned to study the effect of diabetes mellitus in the human not only on respiration but also on phosphorylation reactions of isolated mitochondria, those substrates were chosen that AZZONE and associates [1] had demonstrated gave highest respiratory control, viz. glutamate and pyruvate + malate. The latter substrate was used for the study of respiratory control both in the presence and absence of ATP, which could be thought to mask a true respiratory control by furnishing phosphate acceptor to the phosphorylating system by the action of ATP:ases [1]. Albumin was added to a flask with otherwise similar contents to trap fatty acids possibly present, and thereby making possible an evalution of fatty acid effects in the system.

No differences between non-ketotic diabetics and controls were found in any of the reactions measured. Neither were any correlations found between different blood parameters of the metabolic state of the patients and mitochondrial reactions, nor between degree of diabetic angiopathy or duration of diabetes and mitochondrial metabolism.

This lack of differences must, however, be interpreted with some caution. The wide range of, among other things, age and clinical condition in the present material might open up possibilities for variations of factors that are hard or perhaps impossible to control. It was thus found in patients of high age with atrophic muscles that a lower yield of respiring protein was obtained in the mitochondrial sediment in the isolation procedure. For this reason older patients were excluded, and in the remaining material no variation between age and mitochondrial reactions or mitochondrial protein yield could be found. It is, however, still possible that contamination of non-respiring protein also occurred in the mitochondrial sediment from some of the younger patients, even if histological examinations gave no indication of fibrous, atrophic changes and the frequency of angiopathy in these muscles was low. To what extent such factors might have hidden differences in, e.g. rate of oxidation of pyruvate, found in experimental diabetes [11], where standardization of the material investigated is easier, is not possible to deduce.

Furthermore, there was an increased ATP:ase activity in the basal state in some patients with high

levels of FFA. A considerable part of this activity was not inhibited with oligomycin. This suggests that part of the ATP:ase activity was not of mitochondrial origin [16], and might be an expression of impurities in the form of other cell constituents, in the mitochondrial preparation. This contamination did not seem, however, to be of such magnitude as to be demonstrable in the yield of protein in the mitochondrial sediment in relation to original muscle biopsy weight.

A contamination of the mitochondrial sediment with material that contained ATP:ase might explain the high "resting" respiration of the mitochondria of the severely ketotic patient. The fact that in the absence of ATP the resting respiration was still the highest observed might, however, be an indication of a true derangement of mitochondrial metabolism. This is in agreement with the results of VESTER and STADIE [26] and HALL et al. [13] in the experimental animal. Albumin corrected the elevated resting respiration in the absence of ATP in the patient in question, bringing it back to the normal range, possibly indicating fatty acids as a responsible factor.

In spite of the discussed difficulties in standardization of preparations and the effects of this on comparisons between populations with considerable overlapping of the parameters to be compared, certain conclusions seem possible from the results presented. Phosphorylation reactions measured as respiratory control, ATP:ase activity or P/O ratios do not seem to be deranged in well controlled diabetes mellitus in the human. Furthermore, a high rate of respiration in the presence of a non-limiting amount of phosphate acceptor can be obtained in diabetic patients both with glutamate and pyruvate plus malate as substrate. A decreased oxidation of pyruvate in the mitochondria of ketotic diabetic patients, such as has been described in the experimental animal [11], is by no means excluded by the present work; but it has not been possible to study this problem any further with available techniques.

Acknowledgements. Drs. LENNART ANGERVALL and JOHAN SÄVE-SÖDERBERG, Department of Pathology I, Sahlgrenska sjukhuset, Göteborg, performed the histological examinations. The work was supported by a grant (W 330) from the Swedish Medical Research Council.

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