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OXIDATIVE PHOSPHORYLATION AND RESPIRATORY CONTROL IN MITOCHONDRIA FROM NORMAL, ADENOMATOUS, AND HYPERPLASTIC THYROID GLANDS *

By ROGER W. TURKINGTON and BARBARA NORDWIND

(From the Department of Medicine, Harvard Medical School, and the Thyroid Unit of the Department of Medicine, Massachusetts General Hospital, Boston, Mass.)

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Mitochondria, as the main site of respiration and the conservation of oxidative energy through the formation of adenosine triphosphate (ATP), exert an important controlling influence on the pattern of cellular metabolism. This phosphorylation system defines to a considerable degree the thermodynamic efficiency of the cell and serves as a sensitive regulator of energy turnover, gearing oxygen consumption and energy output to energy demand as represented by the cellular concentration of adenosine diphosphate (ADP) (1–3). Despite the vast amount of experimental work with isolated mitochondria on which the concepts of oxidative phosphorylation and respiratory control are based, there has been relatively little application of these concepts to clinical metabolic research. We are aware of no such observations on mitochondria isolated from thyroid tissue.

The purpose of the present work was to investigate several well characterized mitochondrial properties in intact mitochondria isolated from normal human thyroid tissue and to compare these properties with those of mitochondria from thyroid adenomas and from hyperplastic thyroid glands. Studies were done on sheep thyroid mitochondria as a preliminary to these investigations. The properties studied were the capacity to carry out oxidative phosphorylation and the ability to show respiratory control by adjusting the rate of respiration to the presence or absence of a terminal phosphate acceptor. The metabolism of various Krebs cycle intermediates was observed in sheep thyroid mitochondria. The respiratory rate during oxidation of glutamate was compared in the various human specimens.

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SUBJECTS AND METHODS

Human thyroid tissue was obtained from patients undergoing surgery for thyrotoxicosis or for suspected carcinoma. All but Patients 11 and 12 (Table I) were females. All from whom hyperplastic tissue was obtained had been thyrotoxic clinically and by tests of thyroid function. All had been controlled with propylthiouracil except Patient 15, who showed signs of recurring thyrotoxicosis just prior to surgery. Except for this patient, all were clinically euthyroid at the time of operation. The normal tissue used was the paranodular tissue removed at hemithyroidectomy because of the presence of a discrete nodule. The thyroid tissue was chilled in packed ice immediately after excision, and the experimental procedure was begun after taking representative sections for histological study.

Thyroid glands were obtained from freshly slaughtered sheep at the abattoir and were brought to the laboratory packed in ice.

The mitochondria were isolated in a homogenizing medium containing 0.25 M sucrose, 0.05 M Tris buffer (pH 7.4), 0.001 M sodium ATP, 0.005 M magnesium sulfate, and 0.001 M sodium EDTA. The last three agents were added to prevent the swelling of mitochondria induced by such agents as thyroxine (4, 5), calcium ion (6), inorganic phosphate (6, 7), or reduced glutathione (8) in the homogenate (5, 9). The chilled tissue was quickly sliced and immersed in the ice-chilled isolation medium, minced with fine scissors, and homogenized in 3 to 4 vol of the same medium in a loosely fitting all-glass Potter-Elvehjem homogenizer. Ten to 15 passes by hand were used for this initial homogenization. Additional homogenization for greater mitochondrial yield was not attempted lest there be mechanical damage to the mitochondria. All operations were carried out at 0 to 2° C.

Fractionation of the homogenate was by the method of Schneider (10). A centrifugal force of $600 \times G$ was maintained to remove the nuclei and unfractionated residue and a force of $8500 \times G$ for the centrifugation of the mitochondria. The mitochondrial sediment was resuspended in the isolation medium, recentrifuged as above, and the washing medium discarded. The fluffy coat was carefully removed in this first washing and, since no further separation of the fluffy coat from the mitochondria was observed on subsequent washing, only

a single washing was used. The surface of the tightly packed pellet was rinsed several times with 0.25 M sucrose to remove the Tris-sucrose medium, and the pellet was then gently resuspended in 0.25 M sucrose to contain mitochondria from 5 g of thyroid tissue per ml of suspension.

Mitochondrial respiration was measured by the standard Warburg technique (11). Each manometer vessel contained in a 3.0-ml vol: 3 μmoles ATP, 10 μmoles DPN, 60 μmoles potassium phosphate buffer, 10μmoles magnesium sulfate, 50 μmoles potassium glutamate as respiratory substrate, 0.022 μmole cytochrome C, 0.5 ml mitochondrial suspension added just before incubation and, where indicated, 100 μmoles glucose and an excess of yeast hexokinase. Final osmolarity was adjusted to 270 mOsm with sucrose; the pH of all reagents was previously adjusted to 7.4. After 7 minutes of thermoequilibration a "zero-time" flask was removed and respiration was followed for 30 to 35 minutes. Aliquots from a zero-time flask and the respiration flasks were analyzed for inorganic phosphate by the method of Fiske and Subbarow (12). The amount of phosphate used during the respiration period was calculated in micromoles and this value compared with oxygen uptake in microatoms to obtain the P:O ratio. The Q_{O_2} value was calculated as microatoms of oxygen consumed per 20 minutes per 6.0 mg of mitochondrial protein, as determined on a trichloroacetic acid precipitate by the method of Lowry, Rosebrough, Farr and Randall (13). The respiratory control ratios were determined as the ratio of the respiratory rate in the presence to that in the absence of phosphate acceptor, as supplied by the addition of glucose and hexokinase. It was possible to obtain enough thyroid tissue at surgery to do most, but not all, incubations in duplicate.

ATP, cytochrome C, and hexokinase (step 3a) were all Sigma Chemical Company products. Other materials were reagent-grade commercial products. All reagents and solutions were prepared in water purified by passage through ion exchange columns followed by distillation in a borosilicate glass still.

RESULTS

Studies on sheep thyroid mitochondria. Figure 1 represents the respiration of mitochondria pooled from ten sheep thyroid glands in the presence of various Krebs cycle intermediates. Oxygen uptake varied slightly in absolute amount from batch to batch in four experiments, but the relative rates obtained with these substrates were consistent from one preparation to another. Respiration with citrate varied from zero to slight in the presence of DPN as cofactor but was much enhanced in the presence of TPN. This suggests that oxidation of citrate by thyroid mitochondria proceeds through a TPN-dependent step, as indicated by the studies of Dumont (14) on sheep thyroid homogenates and as has been

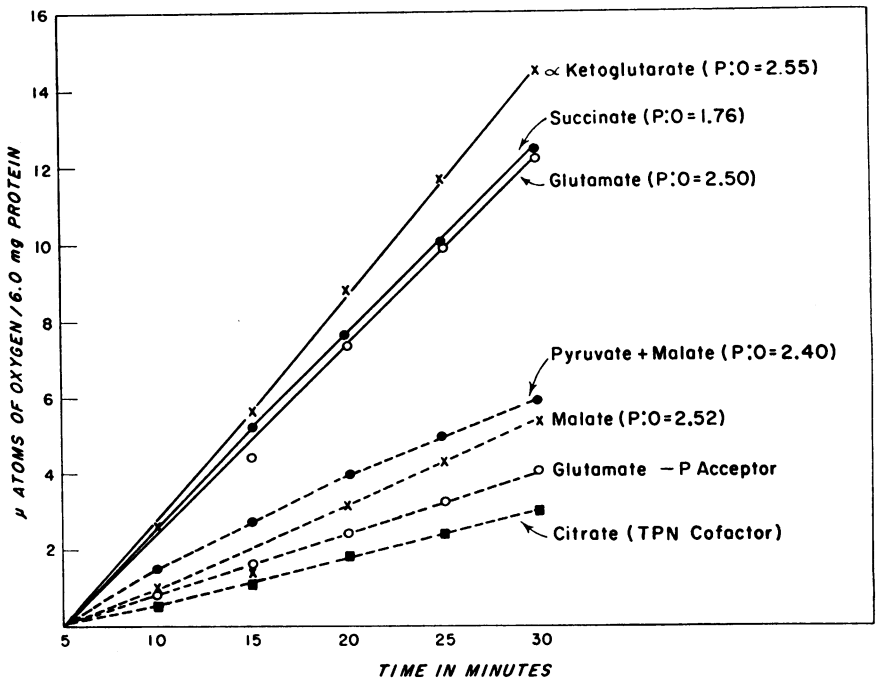

FIG. 1. RESPIRATION OF SHEEP THYROID MITOCHONDRIA IN THE PRESENCE OF VARIOUS SUBSTRATES.

TABLE I

Patient	Age	Clinical diagnosis	Histological description	P:O	Respiratory control ratio	Q_{O_2}
1	38	Nontoxic nodular goiter	Normal thyroid tissue	2.33	3.00	6.6
2	21	Nontoxic nodular goiter	Normal	2.42	2.96	8.3
3	23	Nontoxic nodular goiter	Normal	2.36	3.47	7.0
4	35	Nontoxic nodular goiter	Normal	2.20	1.97	4.8
5	41	Nontoxic nodular goiter	Normal	2.48	3.62	4.2
6	21	Nontoxic nodular goiter	Normal thyroid, 20% discrete follicular adenoma showing hyperplasia	2.06	2.35	7.4
7	68	Nontoxic nodular goiter	Colloidal follicular adenoma	1.61	1.16	3.2
8	22	Nontoxic nodular goiter	Follicular adenomatous nodule	1.0	1.0	2.5
9	77	Nontoxic nodular goiter	Relatively hyperplastic follicular adenoma	2.12	1.0	4.3
10	40	Nontoxic nodular goiter	Well differentiated follicular adenoma, some hyperplasia; fetal-like areas of solid tissue	1.74	1.18	4.1
			Normal paranodular tissue with focal adenomatous growth	2.03	2.30	6.2
11	56	Nontoxic nodular goiter	Colloid adenoma, very large follicles with low epithelium	1.67	1.00	2.9
12	44	Substernal thyroid mass	Involuted colloid adenoma, areas of calcification	1.93	1.60	1.2
13	47	Nontoxic nodular goiter	Involuted colloid follicular adenoma, flat epithelium	1.52	1.08	3.2
14	58	Toxic nodular goiter	Follicular adenoma showing hyperplasia	1.88	1.22	4.4
			Marked diffuse hyperplasia	2.35	1.47	8.1
15	27	Graves' disease	Patchy hyperplasia with areas of involution	2.24	2.50	7.1
16	6	Graves' disease	Diffuse marked hyperplasia, some areas of early involution	2.03	1.68	18.4
17	25	Toxic diffuse goiter		2.50	1.65	8.8
18	14	Toxic diffuse goiter	Focal hyperplasia, early involution	2.26	1.80	6.1
19	6	Graves' disease	Marked diffuse hyperplasia, some colloid areas	2.13	1.73	6.5

shown to be the case in liver and heart mitochondria (15). Respiratory control ratios with the glutamate substrate varied from 2.5 to 3.0, approximating those values reported below for normal human thyroid tissue. Respiration in the absence of a terminal phosphate acceptor is designated "–P Acceptor" in Figure 1. Figure 1 also lists the P:O ratios obtained with each substrate. These values compare favorably with those found in other mammalian tissues (16, 17). In four separate runs with four duplicate incubations in each, the P:O ratio for succinate ranged between 1.55 and 1.97, with an average of 1.76; that for glutamate ranged between 2.22 and 2.70, with an average of 2.50. A P:O ratio for the one-step oxidation of citrate to oxalosuccinate or α-ketoglutarate was not determined.

Oxidative phosphorylation and respiratory control in human thyroid mitochondria. Table I lists the experimentally determined P:O ratios for mitochondria isolated from normal human thyroid tissue, adenomas, and glands showing varying degrees of hyperplasia upon microscopic examination. In every case glutamate was the respiratory substrate. The normal glands showed P:O ratios averaging 2.36 $\pm$ 0.09 (SD), and the hyperplastic glands had P:O ratios that were not significantly different from normal (mean = 2.25 $\pm$ 0.15). None of the values found for the adenomas was in the normal range, the mean value

being 1.68 ± 0.31. This decrease is significant at the 1.4 per cent level (Student's t test). Two glands (Patients 6 and 10) contained a combination of normal and adenomatous tissue, and these mitochondria had P:O ratios intermediate between the normal values and the depressed values of the adenomas.

The capacity for respiratory control in these isolated mitochondria was tested according to the method of Lardy and Wellman (18) by measuring the relative increase in respiratory rate occurring in the presence of glucose, an excess of yeast hexokinase, and a catalytic amount of ATP. As shown in Figure 2, the rates of respiration with and without a phosphate acceptor system to regenerate ADP differed markedly among the mitochondria from normal, adenomatous, and hyperplastic glands. The respiration of the normal mitochondria in the absence of the phosphate acceptor system was relatively low but was stimulated threefold in the presence of hexokinase plus glucose. The mitochondria from the hyperplastic glands showed a relatively high rate of respiration in the absence of phosphate acceptor, and this respiration was stimulated only 65 per cent by the addition of the ADP-regenerating system. The adenomas showed a low rate of respiration in the absence of the phosphate acceptor, but this rate was not significantly stimulated by the addition of the phosphate acceptor system. The

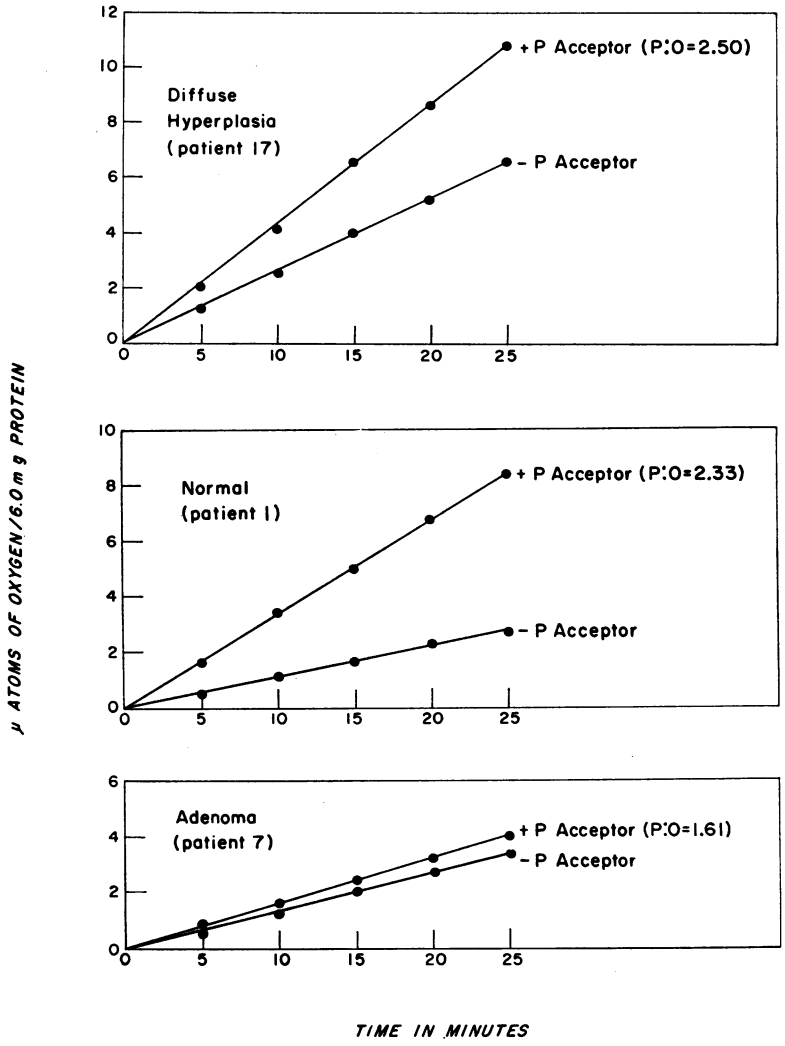

FIG. 2. RESPIRATORY CONTROL AND PHOSPHORYLATIVE EFFICIENCY IN HUMAN THYROID MITOCHONDRIA.

respiratory control ratios are listed for each gland in Table I. The mean normal respiratory control ratio was 3.0 ± 0.57; the mean value of the hyperplastic group was depressed to 1.67 ± 0.15 ($p = 0.01$) (excluding the borderline case of Patient 15). The mean value for the adenomatous group was 1.16 ± 0.19 ($p = 0.001$).

The Qo_2 values listed in Table I were consistently depressed in the adenomatous group, the mean value being $3.2 \pm .7$ μatoms O_2 per 6.0 mg protein per 20 minutes as compared with the normal mean of 6.2 ± 1.5 ($p = 0.035$). The mitochondria from the normal plus adenomatous tissue did not show this depression. Mitochondria from the adenoma of Patient 14 showed a decrease in respiratory activity compared with the normal to high value found in the adjacent hyperplastic tissue. The mean Qo_2 of the hyperplastic group was 9.2 ± 4.2 (not significantly elevated). No explanation is apparent for the marked elevation in the respiratory rate of the mitochondria from Patient 16. Although Barker (19) has demonstrated a consistent increase in respiratory rate of tissue slices taken from thyrotoxic animals, this patient's thyrotoxicosis had been controlled for 3 months with potassium iodide and propylthiouracil.

DISCUSSION

Our findings indicate that there is a basic abnormality in the capacity for respiratory control in mitochondria isolated from hyperplastic thyroid tissue and thyroid adenomas. The adenoma mitochondria show a relative uncoupling of phosphorylation from respiration, as indicated by their lower P : O ratios. The basic mechanism of this uncoupling is not apparent from these studies, but presumably the uncoupling of phosphorylative steps from electron transfer in the adenoma mitochondria accounts, at least to a large extent, for the relative unresponsiveness to ADP as a respiratory stimulant. A factor that cannot be ruled out is the ATPase activity, which may be elevated enough to produce at this rate of respiration a significant hydrolysis of ATP to ADP and an apparent decrease in phosphate consumption with respiration. The presence of an uncoupling mitochondrial factor similar to the mitochrome factor of Polis and Shmukler (20) and Hülsmann, Elliott and Rudney (21), or the "U factor" of Lehninger and Remmert (22), might conceivably be involved here.

Results of studies on the oxidative phosphorylation and respiratory control of mitochondria isolated from various animal tumors have varied with the tumor studied and the experimental conditions. Kielley (23) found normal levels of oxidative phosphorylation in mitochondria isolated from transplantable mouse hepatomas (P : O ratios of 2.22 with α-ketoglutarate and 1.55 with succinate as compared with 2.42 and 1.58, respectively, in normal mouse liver) and respiring in the absence of ATPase-inhibiting fluoride. Yet the phosphorylative ability of these tumor mitochondria was remarkably labile and was lost completely after aging for 24 hours at 0° C or for 25 minutes at 28°, whereas the normal liver mitochondria lost only 30 to 40 per cent of their phosphorylative ability after 24 hours at 0°. It may be that the relatively uncoupled phosphorylation characteristic of the tumor mitochondria in the present study represents a more labile system than exists in the normal mitochondria. Although Williams-Ashman and Kennedy (24) demonstrated oxidative phosphorylation in Jensen sarcoma, the Walker 256 carcinosarcoma, and a mouse amelanotic melanoma, these investigators found that fluoride was obligatory to inhibit the intense ATPase activity present in their system. Emmelot and Bos (25) similarly found a high ATPase activity in rat testicular, ovarian, and adrenal cortical tumors, but this could be inhibited with fluoride to produce high P : O ratios with β-hydroxybutyrate.

Aisenberg (17, 26) has reported P : O ratios, respiratory control ratios, and respiratory activity of mitochondria isolated from five animal tumors. These properties were similar to those of six normal rat tissues, although fluoride was included in the incubations. Studies on mitochondria of Ehrlich's ascites tumor cells by Chance and Hess (27), however, showed no insufficiency in oxidative phosphorylation or respiratory control in these tumor mitochondria. Presumably the decreased rates of respiration of the mitochondria isolated from the adenomata represent a decreased activity of glutamic dehydrogenase in this cell fraction.

The pattern found in the hyperplastic glands is a phosphorylation which is relatively "loosely

coupled" to respiration in comparison to the "tight coupling" found in the normal. Phosphorylative efficiency is maximal or near maximal, as expressed by the P:O ratios found, but respiration is relatively high in the complete absence of a phosphate acceptor system. Thus there is a dissociation of phosphorylative efficiency and respiratory control such that these mitochondria show diminished susceptibility to control by the regenerated ADP, whereas there is no apparent interference with the thermodynamic efficiency of the mitochondria in carrying out their energy-conserving phosphorylation reaction. Hoch and Lipmann (28) have shown in mitochondria isolated from livers of thyrotoxic animals that the phosphate acceptor effect was almost abolished, although the P:O ratio remained unchanged. Chance and Williams (29) have produced a similar loose coupling in isolated rat liver mitochondria by using 3,5-diiodo-4-hydroxybenzoate to depress the respiratory control ratio while the P:O ratio remained unchanged. Baltscheffsky (30) has shown that in the magnesium-deficient system a similar loss of respiratory control occurs under conditions that do not alter the P:O ratio significantly. The fly sarcosomes described by Sacktor and Cochran (31) are capable of yielding high P:O ratios in the presence of ADP, although they do not require ADP for high rates of respiration. The "R factor" isolated from normal rat liver mitochondria by Remmert and Lehninger (32) is capable of fully releasing the respiration of mitochondrial subfragments from dependence upon ADP as phosphate acceptor without depressing the P:O ratio when ADP is also present. It is conceivable that a similar factor latent in thyroid mitochondria is responsible for the conversion of tightly coupled to loosely coupled phosphorylating respiration in thyroid hyperplasia. It seems that ultimately this loose coupling is based upon an increased ATPase activity in these mitochondria. Such a high ATPase activity could produce a high rate of respiration in the hexokinase-free system by regenerating ADP as needed from ATP. Normal P:O ratios would result as long as the hexokinase-glucose trap could compete successfully against the ATPase for the ATP generated by oxidative phosphorylation.

The studies of Ernster, Ikkos and Luft (33) on human skeletal muscle support the concept that the hypermetabolic state, whether thyrotoxic or idiopathic, is characterized at the mitochondrial level by a loose coupling of phosphorylation to respiration paralleled by an increased endogenous ATPase activity. The present studies seem to indicate that the hypermetabolism of the hyperplastic thyroid gland is also characterized at the mitochondrial level by a loose coupling of phosphorylation to respiration, although no direct measurements have been made on endogenous ATPase activity. The elucidation of the physiological implications of this loose coupling awaits further investigation.

SUMMARY

Oxidative phosphorylation, respiratory control, and the rate at which respiration oxidizes glutamate have been studied in mitochondria isolated from normal, adenomatous, and hyperplastic thyroid tissue removed at surgery.

Mitochondria isolated from adenomas showed depressed P:O ratios, a markedly decreased capacity for respiratory control, and decreased rates of respiration. This pattern indicates a relative "uncoupling" of phosphorylation from respiration in these adenomas. The findings are discussed in relation to data on other tumors.

Mitochondria from the hyperplastic glands showed normal P:O ratios but decreased respiratory control ratios in the presence of normal to high respiratory rates. It is suggested that the hypermetabolism of the hyperplastic thyroid gland is characterized at the mitochondrial level by this pattern of "loosely coupled" oxidative phosphorylation.

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