Mitochondrial NADH dehydrogenase in cystic fibrosis
(fibroblasts/O\textsubscript{2} consumption/calcium/electron transport)

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ABSTRACT We have shown that skin fibroblasts from patients with cystic fibrosis (CF) and from carriers for CF [heterozygotes (HZ)] consume more O\textsubscript{2} than do their controls. When the mitochondrial electron transport inhibitor rotenone was added to the cells, the relative inhibition of O\textsubscript{2} consumption was CF > HZ > controls (P < 0.005 in both comparisons). Because rotenone specifically inhibits NADH dehydrogenase, [NADH: (acceptor) oxido-reductase, EC 1.6.99.3], which is the enzyme of energy-conserving site 1 of the mitochondrial electron transport system, activity and kinetics of this enzyme system were studied in fibroblast homogenates. NADH dehydrogenase activity was equal in cells from the three genotypes. At pH 8.0, affinity of the enzyme for its substrate was CF < HZ = controls; at pH 8.6, affinity was CF > HZ = controls (P < 0.005 for the differences). pH optima for the genotypes were without exception 8.0 (CF), 8.3 (HZ), and 8.0 (control). HZ and control lines were distinguished unequivocally in a blind test on the basis of differences in pH optima. Purified mitochondrial preparations revealed pH optima identical to those found in whole cell homogenates. These data suggest that the mutant gene responsible for CF is expressed in the complex mitochondrial NADH dehydrogenase system.

Since its identification as a distinct entity, cystic fibrosis (CF) has been a genetic and pathogenetic enigma. The disease is transmitted within families as an autosomal recessive trait, affected individuals possessing a double dose of the mutant gene. Biological parents of subjects with CF possess a single dose of the mutant gene and, by definition, are obligatory carriers for the condition. However, carriers are clinically normal and their detection prior to the birth of an affected child has been precluded by the absence of detectable effects of the gene in single dose. Males affected with CF do not reproduce and affected females reproduce rarely. Despite this reduced ability of individuals with CF to have children, CF is a uniquely prevalent lethal single-gene disease in Caucasian populations. The basis for the cardinal signs of CF—chronic pulmonary obstruction, gastrointestinal malabsorption, and the sweat chloride abnormality—and their sequelae has been unexplained. The gene product responsible for these signs and symptoms of CF has been widely sought but not yet identified (1).

Although CF is considered to be a generalized disease of exocrine glands (1–4), a number of laboratories including ours have used skin fibroblasts as a model system for CF studies. Fibroblasts are not exocrine cells but they do secrete their products by mechanisms similar to those operating in exocrine cells (5). In addition, the donor’s genotype is retained in cultured fibroblasts for many generations. For these reasons, skin fibroblasts were chosen as a model for CF studies. Using this cell model, we found that skin fibroblasts from subjects with CF in culture express premature senescence (6, 7). To this precarious aging we related increased intracellular Ca\textsuperscript{2+} which occurs in cells from CF and obligate carriers (6, 8, 9). The site of altered intracellular Ca\textsuperscript{2+} pool was traced to mitochondria (10).

Accumulation of Ca\textsuperscript{2+} by mitochondria is a highly complex and incompletely understood process. Numerous variables are involved. Perturbations of any one of several systems potentially could result in the altered mitochondrial Ca\textsuperscript{2+} pools in CF observed by us. One of the systems related to mitochondrial Ca\textsuperscript{2+} influx consists of the terminal steps of oxidation of metabolites because the uphill accumulation of Ca\textsuperscript{2+} in mitochondria is thought to be driven by a proton gradient generated during electron transport (11, 12). Of the many possible explanations for our previous findings, we reasoned that the increased sequestration of Ca\textsuperscript{2+} in mitochondria from subjects with CF and from carriers might be a reflection of increased electron transport. If this were in fact the case, O\textsubscript{2} consumption would be expected to be increased in these cells. We therefore examined O\textsubscript{2} consumption in cells from CF patients and their age- and sex-matched controls (13).

We report here that (i) cells from CF patients and obligate carriers consume significantly more O\textsubscript{2} than do their respective control cells, (ii) after treatment of the cells with the electron transport site 1 inhibitor rotenone (14), cells from CF patients and carriers consume the same amount of O\textsubscript{2} as do controls [i.e., cells with the different genotypes respond differentially to the inhibitor (15)] and (iii) mitochondrial NADH dehydrogenase [NADH:(acceptor) oxido-reductase, EC 1.6.99.3] energy-conserving site 1 of the electron transport system, the target of rotenone, has different properties in cells from CF patients, obligate heterozygotes, and controls.

MATERIALS AND METHODS

Cell Cultures. Skin fibroblast strains from the upper arm were obtained from subjects with CF and from their age- and sex-matched controls and from obligate CF heterozygote parents and their age- and sex-matched controls (8). Cells were stored in liquid nitrogen at early passages and thawed for use as needed. In all but a few experiments, strains were passage matched. For O\textsubscript{2} consumption studies and mitochondrial preparations, monolayers were grown in 285 X 115 mm roller bottles (Bellco Glass). For NADH dehydrogenase studies, monolayers were grown in 75-cm\textsuperscript{2} flasks (Falcon). Monolayers were cultured in Eagle’s minimal essential medium with Earle’s salts plus L-glutamine (K. C. Biologicals, Inc., Lenexa, KA) supplemented with 10% fetal calf serum and 100 units of penicillin and 100 μg of streptomycin per ml (GIBCO). The cultures were maintained at 37°C in 95% air/5% CO\textsubscript{2}. Cells were harvested with trypsin/EDTA (1:250 trypsin plus EDTA at 0.2 g per liter (GIBCO)].

O\textsubscript{2} Consumption and Rotenone Inhibition. Confluent monolayers of fibroblasts were harvested by trypsin/EDTA digestion. Cells were pelleted by centrifugation at 600 X g for

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5 min and washed with 10 ml of cold Hanks’ balanced salt solution at pH 7.4 containing bovine serum albumin (0.1 mg/ml) and dextrose (10 mg/ml). Aliquots (100 μl) of the washed cell suspension were taken for cell counting in a model ZBI Coulter Counter. The washed cells were again pelleted by centrifugation and resuspended in 1 ml of cold Hanks’ solution. O₂ consumption was measured polarographically on a cell suspension of 1 × 10⁷ cells in a final volume of 3 ml by using an O₂ electrode system (Clark model 53, Yellow Springs Instruments) (10). In additional experiments, rotenone (Sigma) was used to specifically inhibit site 1 of the electron transport system. In these experiments the baseline O₂ uptake rate was determined for each cell line. Prior to removal of the cells from the electrode chamber, 100 μl of 100 μM rotenone was added and O₂ consumption was recorded. Preliminary experiments included the addition of 100 μl of 0.1% KCN to assess the extent of inhibition caused by this mitochondrial electron transport inhibitor.

NADH Dehydrogenase. Monolayers were grown to confluence. At the time of assay they were rinsed with 3 ml of trypsin/EDTA and then 5 ml of trypsin/EDTA was added and the monolayer was incubated at 37°C for 10 min. Cells were removed by gentle shaking and transferred to a centrifuge tube containing 1 ml of fetal calf serum (GIBCO). An aliquot (100 μl) was removed and suspended in 9.9 ml of Isoton II balanced electrolyte solution (CMS, Minneapolis, MN) for cell counting. The cell suspension was centrifuged at 410 × g for 5 min, the supernatant was discarded, and the pellet was washed twice with 0.03 M potassium phosphate buffer (pH 7.6) at 0°C. The cells were suspended in 2 ml of 0.12 M potassium phosphate buffer (pH 7.6) at 0°C. After removal of a 150-μl aliquot for protein analysis (15) the cell suspension was homogenized at 0°C at approximately 10,000 rpm in a PT-10 Polytron (Brinkmann). The resulting homogenate was centrifuged at 1400 × g for 10 min and the supernatant was used for assays. Individual flasks yielded between 1.5 × 10⁶ and 2.5 × 10⁶ cells, the cell and enzyme activity yields from the three genotypes being equivalent.

The oxidation of NADH by homogenates was assayed in an Amino–Bowman spectrophotofluorometer with potassium ferricyanide as an artificial electron acceptor (16). The excitation and emission wavelengths for NADH were 350 and 470 nm, respectively. The reaction mixture consisted of 200 μl of 10 mM potassium ferricyanide (Sigma), 60 μl of 1 mM NADH (Sigma) in 2 mM potassium phosphate buffer, and 2.64 ml of 0.12 M potassium phosphate buffer. NADH concentrations

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**Fig. 1.** O₂ consumption rate by fibroblasts was measured before and after the addition of 4 μM rotenone. The change in O₂ consumption rate caused by rotenone was recorded as percentage of initial O₂ consumption rate. Each point represents inhibition measured on one cell strain.

**Fig. 2.** pH optima for oxidation of NADH by NADH dehydrogenase from whole cell extract of controls (Top), carriers (Middle), and CF (Bottom) cell strains assayed at room temperature. Potassium ferricyanide was used as an artificial electron acceptor. Each curve represents a different cell strain.
varied between 2.5 and 25 μM. Reaction mixture pH varied between 7.9 and 8.8. Reagents were mixed and incubated for 5 min at room temperature. Nonenzymatic reaction from CF subjects (328.7 ± 28.2, n = 6) than in their controls (199.5 ± 22.8, n = 6) and in those from heterozygote subjects (227.7 ± 17.7, n = 8) than in their controls (156.3 ± 15.6, n = 8 (P < 0.005, Student’s t test in both comparisons).

At the dosage used, rotenone inhibited O2 consumption in all cell lines tested but the inhibitory effect was CF > HZ > controls (P < 0.005 in both comparisons, Student’s t test). The inhibitory effect of rotenone on O2 consumption in the three genotypes is shown in Fig. 1.

**NADH Dehydrogenase.** Maximal enzyme activity in cell homogenates occurred at pH 8.6 in CF strains and pH 8.0 in control strains (Fig. 2). The clear and consistent difference in pH optima for NADH dehydrogenase in CF and control strains occurred at all passages tested (Table 1). In strains from oblige heterozygotes and their controls, optimum pH was 8.3 in the carriers and 8.0 in their controls (Fig. 2).

pH optima repeatedly were different in the three genotypes. In a blind experiment, carriers could be distinguished from their controls on the basis of this variable alone (Fig. 3).

The effect of pH on NADH dehydrogenase activity in relatively pure mitochondrial preparations is shown in Fig. 4. A sharp peak occurred at pH 8.0 in the control strains and at pH 8.6 in the CF strain. Peak activity in the heterozygote strain was at pH 8.3, but the peak was broad. When these experiments were repeated several times with different cell strains they gave consistent results. Under the conditions used Vmax was equal in the three genotypes. Apparent affinity of the enzyme for its substrate (Km) was determined for each cell line at pH 8.0 (the control optimum) and at 8.6 (the CF optimum). No difference in Km between control and HZ strains occurred at either pH. At pH 8.0, Km for the CF strains was significantly greater and at pH 8.6 it was significantly lower than in the HZ and control strains (P < 0.005 in all comparisons, Student’s t test) (Table 1).
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Table 1. Fibroblast strain source and NADH dehydrogenase kinetics

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DISCUSSION

Because previously reported findings suggested that increased intracellular Ca$^{2+}$ pools and O$_2$ consumption occurred in mitochondria from CF and HZ strains and because cell strains of the three genotypes responded differently to rotenone, an inhibitor specific to site 1 of the mitochondrial electron transport system, we examined kinetics of mitochondrial NADH dehydrogenase, the site 1 enzyme. Consistent and significant kinetic differences in NADH dehydrogenase from CF, HZ, and control cell strains were observed. Enzyme from relatively pure mitochondrial preparations revealed the same pH optima differences in the three genotypes as was found in whole cell homogenates. pH optima in microsomal preparations (data not shown) did not coincide with those from whole cell extracts and did not differ among the three genotypes. We conclude, therefore, that the kinetic differences observed reflect mitochondrial derived enzyme.

Alterations in $K_m$ and pH optima of an enzyme ordinarily suggest protein variants (17) such as those usually associated with Mendelian traits. The NADH dehydrogenase system of mitochondria, however, is so complex, "perhaps the most complex of all flavoproteins" (18), that analogy to other proteins may be inappropriate. In the preparations used here, no effort was made to remove membrane phospholipids which are integral to functioning mitochondrial NADH dehydrogenase (19). Theoretically, therefore, the kinetic differences observed could be a reflection of altered amounts or types of mitochondrial phospholipid in cells of the three genotypes. In any case, the consistent difference in pH optima for NADH dehydrogenase activity between HZ (clinically normal in every way) and their controls precludes any pathological or therapeutic effect of CF as being responsible for the differences reported.

NADH Dehydrogenase. This system, the first of several energy-conserving sites in mitochondrial electron transport, has been extensively studied and reviewed (18–20). We are not aware of a previous study of it in human fibroblasts. The enzyme system has been studied thoroughly in preparations from beef heart mitochondria and, to a lesser extent, in yeast (19). Despite the considerable literature on this system, the mechanism of NADH oxidation, the functional organization of complex I in the inner mitochondrial membrane, and its molecular structure remain obscure (20). Ubiquinone is the natural acceptor for electrons released in the oxidation of NADH, and ferricyanide is the most efficient artificial electron acceptor. The particular preparation of the enzyme which includes mitochondrial inner membrane phospholipid is thought to most closely resemble the in vivo system and has been called complex I of the mitochondrial electron transport system (19). Complex I contains also FMN, iron-sulfur groups, and substantial amounts of ubiquinone 10 (21) and has a molecular weight of approximately 8–8.5 x 10$^6$ (19). The number of different proteins comprising complex I has been controversial. Hatefi and Stigall (19) consider complex I to be a multienzyme complex. Singer and Edmonson (18) and Ragag (20) suggest that complex I is a multisubunit enzyme.

Despite the complexity and controversy that exist there are several important points of agreement concerning mitochondrial NADH dehydrogenase: (i) it is the site of entry of NADH into the respiratory chain; (ii) it catalyzes the dehydrogenation of NADH generated through oxidation of numerous NAD$^+$-linked dehydrogenase reactions; (iii) it is the first of three energy-conserving sites where ATP is formed; (iv) it is one of three sites of uphill Ca$^{2+}$ influx into mitochondria; and (v) it is specifically inhibited by several agents including rotenone (18–21).

Pathogenesis of CF. The earliest lesion in exocrine tissues in CF is the microobstruction of glandular tissue by cellular products that are less soluble than those produced by normal cells (1–4). Delayation of ducts (the "cysts") and fibrosis ensue. These cystic and fibrotic lesions in the pancreas lead to failure of delivery of digestive enzymes to the gut and, in the lung, to chronic obstruction and infection. The potential role of Ca$^{2+}$ in the production of exocrine products of decreased solubility has been suggested and demonstrated (22). The source of this increased Ca$^{2+}$, however, has not been identified. Data from this laboratory suggest that cells from subjects with CF sequester greater amounts of Ca$^{2+}$ in their mitochondria, the major Ca$^{2+}$ sequestering organelle of many cell types (12, 23, 24). Mitochondrial Ca$^{2+}$ stores are thought to be the most important
source of cytosolic Ca\textsuperscript{2+} in eukaryotic cells (23). Secretory granules in exocrine glands have been shown to accumulate and export from cells significant amounts of Ca\textsuperscript{2+} in conjunction with their secretory products (25, 26). Furthermore, it has been reported that parotid acinar cells from patients with CF have significantly higher Ca\textsuperscript{2+} concentrations than do cells from controls (27). It appears reasonable to us to conclude that significantly increased mitochondrial Ca\textsuperscript{2+} could be the source of increased Ca\textsuperscript{2+} in exocrine secretions in CF and that incorporation of increased Ca\textsuperscript{2+} in secretory granules is the basis of decreased solubility of otherwise normal secretory products. Data reported here suggest also that the basis for increased sequestration of Ca\textsuperscript{2+} in mitochondria in CF may be anomalous functioning of NADH dehydrogenase, the first of three sites in the respiratory chain associated with Ca\textsuperscript{2+} accumulation by these organelles.

**Conclusion.** These rationalizations about CF pathogenesis aside, we have traced increased mitochondrial Ca\textsuperscript{2+} and O\textsubscript{2} consumption in CF and HZ fibroblasts to NADH dehydrogenase. We have shown that cells from CF subjects and obligate CF carriers consistently and clearly differ from controls in kinetic characteristics of this enzyme system. We believe that an alteration in a portion of this complex enzyme system may be a fundamental expression of the genetic abnormality in this prevalent lethal disease.

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