Switch to Anaerobic Glucose Metabolism with NADH Accumulation in the β-Cell Model of Mitochondrial Diabetes

CHARACTERISTICS OF βHC9 CELLS DEFICIENT IN MITOCHONDRIAL DNA TRANSCRIPTION*

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To elucidate the mechanism underlying diabetes caused by mitochondrial gene mutations, we created a model by applying 0.4 μg/ml ethidium bromide (EtBr) to the murine pancreatic β cell line βHC9; in this model, transcription of mitochondrial DNA, but not that of nuclear DNA, was suppressed in association with impairment of glucose-stimulated insulin release (Hayakawa, T., Noda, M., Yasuda, K., Yorifuji, H., Kanazawa, Y., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1998) J. Biol. Chem. 273, 20300–20307). To elucidate fully the metabolism-secretion coupling in these cells, we measured glucose oxidation, utilization, and lactate production. We also evaluated NADH autofluorescence in βHC9 cells using two-photon excitation laser microscopy. In addition, we recorded the membrane potential and determined the ATP and ADP contents of the cells.

The results indicated 22.2 μM glucose oxidation to be severely decreased by EtBr treatment compared with control cells (by 63% on day 4 and by 78% on day 6; both p < 0.01). By contrast, glucose utilization was only marginally decreased. Lactate production under 22.2 μM glucose was increased by 2.9- and 3.5-fold by EtBr treatment on days 4 and 6, respectively (both p < 0.01). Cellular NADH at 2.8 μM glucose was increased by 35 and 43% by EtBr on days 4 and 6 (both p < 0.01). These data suggest that reduced expression of the mitochondrial electron transport system causes NADH accumulation in β cells, thereby halting the tricarboxylic acid cycle on one hand, and on the other hand facilitating anaerobic glucose metabolism. Glucose-induced insulin secretion was lost rapidly along with the EtBr treatment with concomitant losses of membrane potential depolarization and the [Ca^{2+}]_{i} increase, whereas glibenclamide-induced changes persisted. This is the first report to demonstrate the connection between metabolic alteration of electron transport system and that of tricarboxylic acid cycle and its impact on insulin secretion.

The mitochondrion has long been one of the main areas of interest for investigators in the field of insulin secretion because it is the primary organelle that metabolizes glucose and other nutrients eliciting insulin secretion. The notion that mitochondria play an important role in insulin secretion has become more feasible with cloning of the ATP-sensitive K⁺ channel/sulfonylurea receptor (1). This is because it is now clear that these channels important for insulin secretion are, in fact, governed by ATP produced mostly by mitochondria.

This importance of mitochondria for insulin secretion is further confirmed by the fact that mutations in mitochondrial DNA are associated with some types of diabetes mellitus (2–4) (for review, see Ref. 5), which is estimated to be the cause of disease in ~1% of people suffering from diabetes (4, 6). Considering that diabetes mellitus is one of the most common diseases, especially in developed countries (e.g. in both the United States (7) and Japan (8) ~10% of people over 40 have this disease), the significance of the percentage must not be overlooked.

Recently, we demonstrated (9) that ethidium bromide (EtBr), an inhibitor of DNA/RNA synthesis which more specifically affects transcription and replication of extrachromosomal genetic components (10–12), i.e. mitochondrial DNA in animal cells, suppresses the transcription of mitochondrial DNA by ~90% at a low dosage (0.4 μg/ml) without affecting nuclear gene transcription, thereby resulting in deterioration of glucose-induced insulin release from the murine insulin-secreting cell line βHC9 (13–15). Similar results have been obtained with other cell types (16, 17) and with the use of another compound to inhibit nucleic acid synthesis (18). However, the overall mechanism that elicits such alteration is still poorly understood, especially from a viewpoint of cellular metabolism.

Herein, we investigated the changes in substrate metabolism and stimulus-secretion coupling caused by suppression of the transcription of mitochondrial DNA by EtBr, which exclusively encodes components of the electron transport system (complexes I, III, IV, and V) and mitochondrial transfer RNA molecules that are used for the synthesis of these components. We have found metabolic derangements, such as accelerated anaerobic metabolism with increased lactate production, indirectly caused by suppression of mitochondrial DNA transcription.

Our observations provide insight not only into the physiological importance of mitochondrial function in insulin release from islet β cells, but also into the pathophysiology of mitochondrial diseases, especially diabetes mellitus, resulting...
from dysfunctional mitochondria, and may further suggest clinical approaches to managing this disease.

**EXPERIMENTAL PROCEDURES**

**Materials**

EtBr, glibenclamide, tetrabutylammonium hydrogen sulfate, fura-2 acetoxyethyl ester, and sulfinpyrazone were purchased from Sigma. Nitrendipine was obtained from Research Biochemicals, norepinephrine from Fluka. Glibenclamide and nitrendipine were dissolved in dimethyl sulfoxide (with final concentrations up to 0.1%), and the same final concentration of dimethyl sulfoxide was added to the controls when they were used.

**Cell Culture**

βHC9 cells were cultured in Dulbecco’s modified Eagle’s medium containing 25 mmol/liter glucose, 1 mmol/liter pyruvate, 15% horse serum, 2.5% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 95% air plus 5% CO2 atmosphere. Supplemental pyruvate was added to ensure cell growth, as with p2 cells (cells lacking mitochondrial DNA) (19). Cells were plated at a density of 0.1–0.15 × 10^5/cm². For the treated cells, 0.4 μg/ml EtBr was added to the culture medium 22–26 h after plating. This EtBr concentration was chosen based on the observation that culturing βHC9 cells with 0.4 μg/ml EtBr for 4 h resulted in a 60% decrease in the level of mitochondrial DNA transcription without changing its copy number (9). Cells at passages from 25 to 38 were used for the experiments.

**Insulin Secretion and Insulin Content**

**Static Incubations**—Insulin secretion experiments were performed as described previously (15). Briefly, four (occasionally three) 16-mm-diameter wells containing cells were used for one test situation. Cells at a concentration of 0.2–0.3 × 10^5 cells (0.1–0.15 × 10^5/cm²) were plated in each 16-mm-diameter well in a 24-well plate. Four (or occasionally 3) of the wells treated with or without EtBr were used for one test situation (usual cell concentrations for control wells at the time of the experiments were 0.5–1.0 × 10^5 cells). In all experiments, cells were preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate (KRB) buffer solution (pH 7.4) composed of (in mmol/liter) 129 NaCl, 5 NaHCO₃, 4.8 KCl, 1.2 KH₂PO₄, 2.0 CaCl₂, 1.2 MgSO₄, and 10 HEPES (pH 7.4) containing 0.1 glucose and 0.2% bovine serum albumin before incubation. Then the medium was removed, and 1 ml of fresh KRB buffer (0.1 mM glucose) containing test substances was introduced. Incubation under the test conditions was then carried out for the indicated times (usually 60 min) at 37 °C. At the end of each incubation, media were sampled and centrifuged, and the supernatants were used for insulin radioimmunoassay. Rat insulin was used as the standard. For determination of insulin content in the cells, each well was suspended in KRB buffer (0.1 mM glucose) containing test substances was introduced. Incubation under the test conditions was then carried out for the indicated times (usually 60 min) at 37 °C. At the end of each incubation, cells were harvested using 1 ml of diethyl ether, and the ether phase containing trichloroacetic acid was discarded. The step was repeated three times to ensure complete removal of the trichloroacetic acid. The extracellular fluid was introduced into a buffer containing 20 mM HEPES, 3 mM MgCl₂, and KOH, as required to adjust pH to 7.75, and then frozen at −80 °C until the assay. Another set of three wells of cells, with or without EtBr treatment, was trypsinated, detached, and used to assay protein amounts. ATP and ADP contents of the cells were assayed in duplicate by the luminescent method as described previously (21) using an ATP bioluminescence assay kit (Sigma). For measurement of the sum of ATP + ADP, ADP was first converted into ATP as described previously (21), and ADP levels were calculated by subtraction.

**Glucose Utilization**

Glucose utilization by βHC9 cells was measured as described previously (22)–(24) with modifications based on 1-([5-³H]glucose. In brief, ~0.5 × 10^5 cells dispersed by trypsinization were first incubated for 30 min in 1 ml of KRB buffer containing 0.1 mM glucose (preincubation). At the end of the preincubation, the cells were briefly centrifuged, supernatants were aspirated, 100 μl of KRB buffer containing 1 μCi of 1-([5-³H]glucose (Amersham Biosciences, specific activity 16.5 Ci/mmol) was introduced, and incubation was continued for another 60 min. Then, the reaction was terminated by adding 10 μl of 3% HCl and 40 μl of ethanol to the incubation mixture. Formed H₂O was equilibrated with 0.5 ml of H₂O in outer vessels, as described previously (23), for 24 h at room temperature. The glucose concentration (radioactive and nonradioactive combined) was set at 0.8 or 22.2 mM during the incubations.

**Glucose Oxidation**

Glucose oxidation by βHC9 cells was measured as described previously (22, 24, 25) by using uniformly labeled 1-[¹⁴C]glucose with modifications. In brief, ~0.5 × 10^5 cells dispersed by trypsinization were first incubated for 30 min in 1 ml of KRB buffer containing 0.1 mM glucose (preincubation). At the end of the preincubation, the cells were held in a spectrophotometer (PerkinElmer Life Sciences LS-5). The cell suspension in each cuvette was stirred continuously with a small magnetic bar within the cuvette during each experiment. The temperature of the cell suspension was maintained at 37 °C by circulating warm water through the cuvette holder. An excitation wavelength of 340 nm and an emission wavelength of 510 nm were used for the measurement. In some cases, an excitation wavelength of 380 nm was used for the reciprocal monitor and, to confirm the Ca²⁺ dependence of the fluorescence, the [Ca²⁺]ᵢ, was calculated as described previously (15) employing values of auto- and extracellular fura-2-εmitted fluorescence, which were determined using two of the four cuvettes applied for each experiment. For stimulation, a 100-fold concentration of the stimulus was added, gently, as an injection.
briefly centrifuged, the supernatants were aspirated, 100 μl of KRB buffer containing 1 μl of n-[1-14C]glucose (PerkinElmer Life Sciences, specific activity 13.0 mCi/mmol) was introduced, and incubation was continued for another 60 min. Then, the reaction was terminated by adding 200 μl of 0.1 N HCl to the incubation mixture. Formed 14CO2 was trapped by 300 μl of 1 M benzenthionyl hydroxide (Sigma) in methanol for 48 h at room temperature. The glucose concentration (radioactive and nonradioactive combined) was set at 0.8 or 22.2 mM during the incubations.

**Lactate Production and LDH Activity**

Lactate production was measured by the lactate oxidase method using a Determiner LA assay kit (Kyowa Medics, Tokyo, Japan). Five 30-mm-diameter dishes of cells were used for one test condition. Cells at a concentration of 0.7–1.1 × 10^5/dish (0.1–0.15 × 10^6/cm²) were plated onto the 30-mm-diameter dishes. After the indicated culture periods, the cells were preincubated for 30 min at 37 °C with 3 ml of a fresh KRB buffer solution (containing 0.1 mM glucose) after being washed twice with the same buffer. Then, the medium was removed, and 3 ml of fresh KRB buffer containing 0.1 or 22.2 mM glucose was introduced. Incubation under the test conditions was carried out for 60 min at 37 °C. At the end of the incubation, media were sampled and centrifuged, and the supernatants were used to assay for lactate released during the incubation period. The cells were trypsinized, detached, and used to assay for protein amount.

LDH activity was measured using LDH assay kit (TaKaRa, Ohtsu, Japan). After the indicated culture periods, cells of four of the 32-mm-diameter wells at a concentration of 1.0 × 10^5/cm² were plated in 100-μl final volume with 1% Triton X-100 were applied for photometric measurement in final volume of 200 μl of assay mixture as indicated by the manufacturer. LDH from rabbit muscle (Wako, Tokyo, Japan) was used as the standard.

**Measurement of NADH Autofluorescence**

NADH autofluorescence (400–500 nm) of the monolayer-cultured cells was imaged using two-photon excitation microscopy (26, 27) at an excitation wavelength of 720 nm. Cells were plated at a density of 0.1–0.15 × 10^6/cm² and cultured with or without EtBr for the indicated times on 5 mm-in-diameter glass coverslips.

**Statistical Analysis**

Data are expressed as means ± S.E. Statistical significance was evaluated by one-way analysis of variance using Bonferroni’s method unless otherwise indicated. For all comparisons in the figures, * and ** denote 0.01 ≤ p < 0.05 and p < 0.01, respectively.

**RESULTS**

**Effect of EtBr Treatment on Insulin Secretion**—In static incubations, during suppression of the electron transport system by EtBr, glucose-induced insulin secretion was decreased by 4-day EtBr treatment and had almost disappeared by day 6, as shown in Fig. 1A. However, glibenclamide-stimulated insulin secretion was not reduced on day 2, 4, or 6 of EtBr treatment (Fig. 1A). The concentration-secretion relationship curves (Fig. 1C) showed a decrease in insulin release with glucose stimulation. These results are similar to our previous data (9). Thus, suppression of the electron transport system ultimately abolished glucose-stimulated insulin secretion (Fig. 1, A and C), whereas insulin secretion elicited by glibenclamide was maintained (Fig. 1A) because it does not employ metabolism for its effect, as discussed previously (9). In addition, the formerly observed increase in basal (nonstimulated) insulin secretion in EtBr-treated cells (9) was also seen in the current series of experiments on most occasions. However, the degree of the increase was much smaller, possibly because of the more dense initial cell concentration (0.03 × 10^6/cm² versus 0.1–0.15 × 10^6/cm²) with which culture was started in this series of experiments.

Perfusion experiments were performed on day 4 (Fig. 1B), and in these experiments, both the first and the second phase of glucose-elicted insulin secretion were reduced, the latter being nearly abolished by the treatment.

**Effect of EtBr Treatment on [Ca²⁺], Increase by Glucose and Glibenclamide**—The [Ca²⁺], measurement was performed on day 4 (Fig. 2). Basal (initial) [Ca²⁺], at the start of stimulation did not differ significantly between EtBr-treated (189 nM; n = 13) and control conditions (212 nM; n = 23) by Student’s t test. Both glucose and glibenclamide increased [Ca²⁺], under control conditions. However, in EtBr-treated cells, the mean ratio over the initial (start of stimulation) [Ca²⁺], was decreased significantly under glucose stimulation at every time point 4 min after stimulation and thereafter. By contrast, the glibenclamide-induced [Ca²⁺], increase did not differ significantly except at 2 min after the start of stimulation.

**Effect of EtBr Treatment on the Membrane Potential Produced by Glucose and Glibenclamide**—Membrane potential was measured by whole cell mode patch clamp on day 6 (Fig. 3). In control cells, both glucose and glibenclamide elicited depolarization of the membrane potential with an action potential burst. Glibenclamide was still able to trigger the potential without being affected by EtBr treatment (Fig. 3D). By contrast, the glucose-induced change in membrane potential was abolished by EtBr treatment, as shown in Fig. 3C.

**Nucleotide Contents of HC9 Cells**—ATP and ADP contents were measured on day 4 and day 6. As shown in Fig. 4, the steady-state ATP and ADP contents of the cells (measured after a 30-min incubation in 0.1 mM glucose after continuous culture) were fairly well maintained even with EtBr treatment, i.e. they were equilibrated. The ATP/ADP ratio before stimulation did not differ significantly on either day 4 or 6 between EtBr-treated and control cells. After glucose stimulation, although no significant ATP increase was observed on either day in EtBr-treated cells or even in control cells, the ADP content was decreased significantly in control cells (days 4 and 6) and in EtBr-treated cells (day 4). As a result, the ATP/ADP ratio was increased significantly (at 30 min) in control cells. In EtBr-treated cells, however, this increase in the ATP/ADP ratio was not significant on either day.

**Glucose Utilization, Oxidation, and Lactate Production**—With 4- and 6-day EtBr treatment, glucose oxidation (Fig. 5B), measured by the rate of 14CO₂ formation from D-[14C]glucose, was decreased severely compared with control cells (by 63% on day 4 and by 78% on day 6; both p < 0.01 by Student’s t test) under the stimulatory (22.2 mM) condition; under the nonstimulatory (0.8 mM) condition, it was substantially decreased as well (p < 0.05 on day 4 and p < 0.01 on day 6; Student’s t test). By contrast, glucose utilization was only marginally affected on both day 4 and day 6 of EtBr treatment, as measured by H2O formation from D-[5-3H]glucose, suggesting the glycolytic pathway to be intact (Fig. 5A). The slight, but significant (Student’s t test), difference in glucose utilization could represent a backward effect of suppressed mitochondrial metabolism (see “Discussion”). In control cells, the ratio of glucose oxidation to utilization under the stimulatory (22.2 mM glucose) condition was 10–15%. This percentage was similar to previous data obtained with HC9 cells (14), which was much smaller than those (~70% of utilization) with mouse (28) and rat (24) islets at a stimulatory glucose concentration (16.7 mM) measured using the same methods for both oxidation and utilization.

Lactate production under basal (0.1 mM glucose) conditions was not different on either day 4 or day 6 (Fig. 5C). At 22.2 mM glucose, it was increased by 2.9- and 3.5-fold by EtBr on days 4 and 6, respectively (both p < 0.01; Student’s t test) (Fig. 5C). The measured LDH activity was 41.1 ± 3.3, 39.3 ± 1.7, and 34.6 ± 0.9 units/mg of protein for 2, 4, and 6 days culture for the control cells, respectively; it was 39.3 ± 3.3, 29.8 ± 4.8, and 15.8 ± 3.8 units/mg protein, for 2, 4, and 6 days EtBr-treated.
cells, respectively. Thus, LDH activity decreased gradually in the EtBr-treated cells during the course of cell culture and finally reached significance ($p < 0.01$) on day 6 to the control.

**NADH Autofluorescence**—We measured the originally very weak autofluorescence of βH9 cells by two-photon excitation microscopy. Because the observed autofluorescence had a granular appearance, as shown in Fig. 6A, it was suggested that this autofluorescence originated mainly from NADH in the mitochondria. As shown in Fig. 6B, cells cultured with EtBr showed a more pronounced autofluorescence image compared with the control cells.

By calculating the autofluorescence of the non-nuclear area (area except the nucleus which is observed as a dark round shape in Fig. 6, A and B), it was revealed that the concentration of NADH was significantly increased in EtBr-treated cells compared with control cells (Fig. 6C); the cellular NADH concentrations at 2.8 mM glucose were increased by 35 and 43% by EtBr treatment on days 4 and 6, respectively (both $p < 0.005$ by Student’s $t$ test).

**Effect of EtBr Treatment on Cell Number and Cellular Insulin Content**—As shown in Fig. 7, EtBr treatment resulted in a decrease in cell number compared with control cells as expressed by the ratio of the cell number of EtBr-treated cells over that of control cells, which became more evident as the culture period became longer. By contrast, the cellular insulin content ratio of EtBr-treated cells to that of control cells increased during the course of cell culture (Fig. 7).

**DISCUSSION**

**Suppression of Electron Transport System**—In this study, we analyzed the relationship between the electron transport system and insulin secretion and also scrutinized the concomitant metabolic derangement, by the application of EtBr to the murine insulin-secreting cell line βHC9. Our previous observations showed that culturing βHC9 cells with 0.4 μg/ml EtBr for 4–6 days results in an ~90% reduction in the level of mitochondrial DNA transcription (9). This reduced transcription level is very likely to give rise to malfunction of the mitochondrial electron transport system with loss of mitochondrial ATP production because mitochondrial DNA encodes subunits of respiratory complexes I, III, IV, and V (complex V, ATP synthase), and mitochondrial transfer RNA molecules that are used for the synthesis of these components (5, 29). At this point, it is important to note that the transfer RNAs encoded by mitochondrial DNA are only used for the synthesis of the above mentioned respiratory chain components encoded by the mitochondrial DNA itself.

In fact, in our present series of experiments, the increase in the ATP/ADP ratio which was observed in control βHC9 cells after glucose challenge was blunted when the cells were cultured with EtBr for 4–6 days (Fig. 4).

**Changes in Insulin Secretion Induced by EtBr Treatment**—Thus, we assumed that suppression of the electron transport system interfered with glucose-induced ATP production, thereby perturbing cellular membrane potential formation with a blunted $[\text{Ca}^{2+}]$, rise, thus ultimately abolishing glucose-stimulated insulin release. In fact, as shown in Fig. 1A, glu-
cose-induced insulin secretion was lost rapidly with EtBr treatment. Depolarization of the membrane potential (Fig. 3) and the $[\text{Ca}^{2+}]_i$ increase (Fig. 2) were also suppressed with this treatment, whereas glibenclamide-induced changes persisted (Figs. 2 and 3). The concentration-secretion relationship curves (Fig. 1C) showed a decrease in insulin release stimulated by glucose. These results indicate that glucose requires mitochondrial metabolism for its stimulatory effect on insulin secretion, which is mediated by membrane depolarization and an increase in the $[\text{Ca}^{2+}]_i$. Interestingly, the first and the sustained second phases characteristic of this cell line (15) were both decreased by EtBr (Fig. 1B). A relative increase in the cellular
insulin content of EtBr-treated cells compared with the control cells (Fig. 7), which was also observed in the previous series of experiments (9), could at least partly be explained by such a decrease in secretion because the culture medium contains a high concentration (25 mM) of glucose; in addition, it may be ascribed to a possible up-regulation of insulin synthesis as discussed previously (9).

In the current study, no significant difference in the basal

**FIG. 4.** Changes in ATP and ADP contents in EtBr (EB)-treated and control βHC9 cells before and after glucose stimulation. ATP and ADP contents were measured on day 4 and day 6 before stimulation (just after preincubation, 0 min) or after glucose stimulation for the indicated times, as described under “Experimental Procedures.” Data presented are the means ± S.E. of three observations for each time point. * indicates 0.01 ≤ p < 0.05, and ** denotes p < 0.01, otherwise the difference is not significant from the time point 0 of each condition. A and B, upper panels, ATP and ADP contents on day 4 (A) and day 6 (B). Lower panels, ATP/ADP ratio on day 4 (A) and day 6 (B).
[Ca$^{2+}$], was observed between EtBr-treated and control cells; in our previous experiment, however, the former had a lower basal [Ca$^{2+}$] than the latter (9). This might be the result of a difference in the materials that were used for measurement, i.e. the cell suspension in the current experiments versus monolayer-cultured cells in the previous measurements where the cell

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**Fig. 5. Effects of EtBr (EB) treatment on glucose utilization, oxidation, and lactate production under basal and stimulatory glucose concentrations in βHC9 cells.** Glucose utilization, oxidation, and lactate production were measured on days 4 and 6 as described under "Experimental Procedures." Data presented are the means ± S.E. Statistical significance was evaluated by Student's t test. * indicates $0.01 < p < 0.05$, ** denotes $p < 0.01$, and NS means there was no significant difference between EtBr-treated and control cells. $n$ (observation number for one column) is 10, 8–10, 7, and 7–8, for glucose utilization on day 4, the same on day 6, glucose oxidation on day 4, and the same on day 6, respectively. A, glucose utilization by βHC9 cells during a 60-min incubation measured by $^3$H$_2$O formation from 1-1H$^2$Hglucose. B, glucose oxidation by βHC9 cells during a 60-min incubation measured by $^1$CO$_2$ formation from 1-1$^1$Cglucose. C, lactate production by βHC9 cells during a 60-min incubation.
cluster used for the measurement was less confluent for EtBr-treated cells than for controls.

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**Steady-state Metabolism in Dysfunctional Mitochondria of EtBr-treated βHC9 Cells**—Regarding the change in glucose metabolism evoked by EtBr, glucose utilization that corresponds to the metabolic flow of the glycolytic pathway was only marginally affected by EtBr treatment on either day 4 or day 6 compared with control cells (Fig. 5A). By contrast, glucose oxidation, which shows tricarboxylic acid cycle activity, decreased dramatically and had almost disappeared on day 6 of EtBr treatment (Fig. 5B). This indicates that EtBr treatment not only suppressed the electron transport system but also secondarily hindered and decelerated the tricarboxylic acid cycle. Why did the tricarboxylic acid cycle decelerate, given that it is not linked directly to the electron transport system itself?

We considered the possibility of this deceleration being caused by intramitochondrial accumulation of NADH secondary to suppression of the electron transport system. In this connection, it is important to point out here that tricarboxylic acid cycle rotation is coupled with several reactions from NAD to NADH, for instance, that from isocitrate to α-ketoglutarate (Fig. 8, A and C). Suppression of the electron transport system by EtBr, which converts NADH to NAD at its first step (NADH dehydrogenase, complex I) (Fig. 8, A and C), should increase the intramitochondrial NADH concentration, which would then decelerate tricarboxylic acid cycle rotation in a retrograde fashion, interfering with enzymatic reactions in the NADH-NAD-coupled steps, eventually impeding the tricarboxylic acid cycle with a resultant decline in CO2 formation (Fig. 8B). To demonstrate this hypothesis, we evaluated intracellular NADH concentrations, which was only possible by applying two-photon excitation microscopy in this cell line, and found the cellular NADH concentration actually to be increased (Fig. 6, B and C).
Mitochondrial Metabolism and Insulin Secretion

Cytoplasmic Anaerobic Metabolism Caused by Suppression of the Mitochondrial Electron Transport System in EtBr-treated βHC9 Cells—An increased intramitochondrial NADH concentration, and imbalance of the flavin coenzymes as well, functionally halt the NADH shuttle system essential for insulin secretion (30) in a retrograde fashion, and as a result, the NADH increase in the mitochondria is spread to the cytoplasm. This NADH increase in the cytoplasm facilitates production of lactate from pyruvate that is coupled with the conversion of NADH to NAD in the cytoplasm. Indeed, on days 4 and 6 of EtBr treatment, lactate production was increased markedly, showing anaerobic metabolism.

Although lactate production was facilitated (Fig. 5C), LDH activity was not up-regulated, but rather decreased by 20–50% in the EtBr-treated cells compared with the controls on the same day. Decreased ATP/ADP (Fig. 4) may have some influence on the stability of the LDH mRNA through production of cAMP from ATP, because it is known that protein kinase A activation very strongly prolongs half-life of mRNA of the enzyme (31).

In addition, as shown by the ratios of glucose oxidation over utilization for control βHC9 cells (10–15%; Fig. 5, A and B) even without EtBr treatment, this cell line may be relatively more dependent for its energy production on glycolysis than on mitochondrial metabolism compared with normal mouse (28) or rat (24) islets. In other words, under our culture conditions, this cell line may be relatively more anaerobic than normal islet tissues, in which glucose oxidation corresponds to ~70% of the utilization at a stimulatory glucose concentration (24, 28). EtBr treatment further enhanced this condition exceedingly, as assessed by the ratio of glucose oxidation to utilization (Fig. 5, A and B) being much smaller than those observed in control cells.

In fact, LDH activity of βHC9 cells was ~50 times of INS1 cells under the control conditions in our hands. This is not surprising, however, considering that 25-fold overexpression of this enzyme displayed only a small (20–30%) decrease in glucose oxidation as shown in INS1 cells (32). Thus, because βHC9 cells originally possess very high LDH activity, we cannot conclude from our data, which are based on this cell line, that EtBr is able to exert a similar effect in normal islet tissues. Actually, we had tested the effect of EtBr in the rat islets and obtained the same kind of results by EtBr on glucose-stimulated insulin secretion, but to a lesser extent, possibly because of a lower LDH activity.²

Summary—In EtBr-treated βHC9 cells, we observed 1) an increase in NADH and decreased ATP production resulting from suppressed transcription (9) of the electron transport system; 2) decelerated tricarboxylic acid cycle flux with decreased CO₂ formation because of accumulated NADH; and, conversely, 3) anaerobic metabolism being favored as shown by increased production of lactate.

In EtBr-treated βHC9 cells, glucose-induced insulin secretion was rapidly depleted such that ATP synthesis and conversion of NADH to NAD must have been impaired because of suppressed transcription of the mitochondrial DNA that en-

² M. Noda, M. Komatsu, and G. W. G. Sharp, unpublished observation.
codes components of the electron transport system and the transfer RNAs that are used for synthesis of these components. However, the remainder of the mitochondrion should have been intact because the mitochondrion is otherwise constituted of proteins encoded by nuclear DNA. In this regard, this model corresponds well to the islets in mitochondrial diabetes mellitus in which a mitochondrial transfer RNA(s) or a component(s) of the electron transport system is damaged because of a mitochondrial DNA mutation.

Moreover, this is the first report to demonstrate the connection between metabolic alteration of electron transport system and that of tricarboxylic acid cycle, which is only possible by applying two-photon excitation microscopy to the measurement of very weak NADH autofluorescence of this cell line. This system of EtBr treatment of an insulin-secreting cell line not only provides insights into the physiological importance of mitochondrial diabetes mellitus, which affects, e.g. in Japan, several tens of thousands of people.

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