Progressive Accumulation of Mitochondrial DNA Mutations and Decline in Mitochondrial Function Lead to β-Cell Failure*

Rebecca A. Simmons‡, Irena Suponitsky-Kroyter, and Mary A. Selak
From the Department of Pediatrics Children's Hospital Philadelphia and University of Pennsylvania, Philadelphia, Pennsylvania 19104

A key adaptation enabling the fetus to survive in a limited energy environment may be the reprogramming of mitochondrial function, which can have deleterious effects. Critical questions are whether mitochondrial dysfunction progressively declines after birth, and if so, what mechanism might underlie this process. To address this, we developed a model of intrauterine growth retardation (IUGR) in the rat that leads to diabetes in adulthood. Reactive oxygen species (ROS) production and oxidative stress gradually increased in IUGR islets. ATP production was impaired and continued to deteriorate with age. The activities of complex I and III of the electron transport chain progressively declined in IUGR islets. Mitochondrial DNA point mutations accumulated with age and were associated with decreased mitochondrial DNA content and reduced expression of mitochondria-encoded genes in IUGR islets. Mitochondrial dysfunction resulted in impaired insulin secretion. These results demonstrate that IUGR induces mitochondrial dysfunction in the fetal β-cell, leading to increased production of ROS, which in turn damage mitochondrial DNA. A self-reinforcing cycle of progressive deterioration in mitochondrial function leads to a corresponding decline in β-cell function. Finally, a threshold in mitochondrial dysfunction and ROS production is reached, and diabetes ensues.

Epidemiological studies have revealed strong statistical links between poor fetal growth and the subsequent development of type 2 diabetes in adulthood (1–6). These associations have led to the “fetal origins of adult disease hypothesis,” which states that “to ensure fetal survival, adaptations to intrauterine deprivation result in a permanent reprogramming of key organ systems” (2). Both insulin action and insulin secretion are impaired in individuals who were growth-retarded at birth (7–12).

Uteroplacental insufficiency, the most common cause of in utero growth retardation, limits the supply of critical substrates such as oxygen, glucose, and amino acids to the fetus and results in poor fetal growth (13–15). This abnormal metabolic intrauterine milieu affects development of the fetus by modifying gene expression and function of susceptible cells, such as the β-cell (16–19). The molecular mechanisms responsible for permanent changes in gene expression are not known, but they are a critical element in our understanding of how factors in early development can lead to long term consequences in aging and disease.

A key adaptation enabling the fetus to survive in a limited energy environment may be the reprogramming of mitochondrial function (17, 18). However, these alterations in mitochondrial function can have deleterious effects, especially in cells that have a high energy requirement, such as the β-cell. The β-cell depends upon the normal production of ATP for nutrient-induced insulin secretion (20–27) and proliferation (28). Thus, an interruption of mitochondrial function can have profound consequences for the β-cell.

Mitochondrial dysfunction can also lead to increased production of reactive oxygen species (ROS), which will lead to oxidative stress if the defense mechanisms of the cell are overwhelmed. β-Cells are especially vulnerable to attacks by ROS, because expression of antioxidant enzymes in pancreatic islets is very low (29, 30), and β-cells have a high oxidative energy requirement. Increased ROS impair glucose-stimulated insulin secretion (28, 31, 32), decrease gene expression of key β-cell genes (33–39), and induce cell death (40–42).

We have developed a model of intrauterine growth retardation (IUGR) in the rat that leads to diabetes in adulthood (13–16) with the salient features of most forms of type 2 diabetes in the human: progressive defects in insulin secretion and insulin action prior to the onset of overt hyperglycemia (16). Decreased proliferation leads to a progressive decline in β-cell mass (19). A critical question is whether mitochondrial dysfunction that is induced by an abnormal intrauterine milieu in fetal cells is permanently interrupted, and if so, what mechanism(s) might underlie this process. Using this model, we have tested the hypothesis that uteroplacental insufficiency disrupts the function of the electron transport chain in the fetal β-cell and leads to a debilitating cascade of events: increased production of reactive oxygen species, which in turn damage mitochondrial DNA (mtDNA) and cause further production of ROS. The net result is progressive loss of β-cell function and eventual development of type 2 diabetes in the adult.

MATERIALS AND METHODS

Animal Model

We have described our surgical methods previously (13–16). In brief, time-dated Sprague-Dawley pregnant rats were individually housed under standard conditions and allowed free access to standard rat chow

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† To whom correspondence should be addressed: University of Pennsylvania, BRB II/III, Rm. 1308, 421 Curie Blvd., Philadelphia, PA 19104. Tel.: 215-746-5139; Fax: 215-573-7627; E-mail: rsimmons@mail.med.upenn.edu.

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‡ To whom correspondence should be addressed: University of Pennsylvania, BRB II/III, Rm. 1308, 421 Curie Blvd., Philadelphia, PA 19104. Tel.: 215-746-5139; Fax: 215-573-7627; E-mail: rsimmons@mail.med.upenn.edu.

The abbreviations used are: ROS, reactive oxygen species; IUGR, intrauterine growth retardation; MOPS, 4-morpholinepropanesulfonic acid; ECL, enzyme-catalyzed chemiluminescence; mtDNA, mitochondrial DNA; HNE, trans-4-hydroxy-2-nonenal; SSCP, single-strand conformation of polymorphism.
and water. On day 18 of gestation (term is 22 days), the maternal rats were anesthetized with intraperitoneal xylazine (8 mg/kg) and ketamine (40 mg/kg), and both uterine arteries were ligated (IUGR). This resulted in the development of 50% reduced rat litters. Rat recovered within a few hours and had ad lib access to food and water. The pregnant rats were allowed to deliver spontaneously, and the litter size was randomly reduced to 8 at birth to assure uniformity of litter size between IUGR and control litters. The pups were fostered to unoperated normal female rats and remained with their foster mothers until they were weaned. Unless otherwise noted, all studies were carried out in littermate islets and all groups of rats were maintained on a constant diet and were housed under ambient conditions to evaluate the evolution of the disease process prior to the onset of diabetes. Only male animals were studied to avoid the potentially confounding hormonal variables associated with female rats.

**Assays**

Blood glucose concentrations were determined in duplicate using the Hemogreen blood glucose analyzer (Angiholm, Sweden). Plasma insulin concentrations were measured in duplicate by radioimmunoassays using rat insulin as the standard (Linco, St. Louis, MO). The within- and between-assay coefficients of variation for the insulin assay were 4 and 10%, respectively.

**Insulin Secretion Studies**

Insulin secretion was measured by standard static incubation methods used at the University of Pennsylvania Diabetes Center and by other laboratories (43). Islets were isolated from IUGRs and controls by collagenase digestion as previously described (44). Islets were cultured for 3 days in RPMI 1640 culture medium containing 11 mM glucose, supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin (Invitrogen). 100 islets were subjected to static incubation experiments under the following conditions: 3.3 mM glucose, 16.7 mM glucose, or 4.8 mM leucine. Briefly, islets were incubated in a shaking water bath for 60 min at 37 °C in Krebs-Ringer bicarbonate buffer containing 129 mmol/liter NaCl, 5 mmol/liter NaHCO3, 1.2 mmol/liter KH2PO4, 2.5 mmol/liter CaCl2, 0.1% bovine serum albumin, 10 mmol/liter HEPES at pH 7.4, 5 g/liter bovine serum albumin, and 25 mM glucose. Islets were then incubated for 120 min in the presence of glucose or leucine plus glucose. Insulin was measured in the incubation medium at 20, 40, 60, and 120 min, and the results were expressed in microunits/ng of DNA and normalized for β-cell mass. β-Cell mass was determined using point-counting morphometry as previously described (19). Insulin concentration was determined by radioimmunoassay using rat insulin as a standard.

**ATP Measurement**

Total cellular ATP was determined in fetal islets immediately after isolation and in postnatal islets after 3 days of culture by chemiluminescence using a reagent based on firefly luciferase. The light was measured immediately after isolation and in postnatal islets after 3 days of culture by chemiluminescence using a reagent based on firefly luciferase. The light was measured immediately after isolation and in postnatal islets after 3 days of culture by chemiluminescence using a reagent based on firefly luciferase. ATP concentration was determined using a kit (Promega). The kit was used to measure ATP concentration in fetal islets immediately after isolation and in postnatal islets after 3 days of culture. The results were expressed in micromoles per milligram of protein.

**Fluorescence Measurement of ROS**

ROS production in islet cells was measured using dichlorofluorescin diacetate (Molecular Probes, Inc., Eugene, OR) fluorescence. The non-fluorescent dye generates a fluorescence signal after reacting with ROS. The fluorescence signal was measured using a fluorescence plate reader with excitation wavelength at 505 nm and emission wavelength at 540 nm. Data are expressed as percent of control.

**Activity of Individual Complexes of the Electron Transport Chain in Islets**

**Complex I**—Complex I (NADH:ubiquinone oxidoreductase) was assayed in freeze-dried islets using the synthetic, short-chain ubiquinone analog Q as the electron acceptor. The suspension was diluted to a protein concentration of ~2 mg/ml in 0.25 M sucrose, 20 mM MOPS, 1 mM EDTA, 5 mM inorganic phosphate, 0.1% bovine serum albumin (fatty acid-free), and 1 mM ADP, pH 7.4. Enzyme activity was determined in the range in which activity was linear with respect to concentration. Complex I activity was determined in the presence of rotenone. Complex I activity was then calculated as the difference between the total rate and the rate in the presence of rotenone.

**Complex II**—Complex II (succinic dehydrogenase) activity was assayed using Q1 as an intermediate electron acceptor and 2,6-dichlorophenolindophenol as a final electron acceptor. The enzyme was contained in the presence of succinate. After activation, 2,6-dichlorophenolindophenol was added. After a short period of temperature reequilibration, Q1 was added to initiate the reaction. Reduction of 2,6-dichlorophenolindophenol was followed. After a linear rate was observed, malonate (a specific complex II inhibitor) was added, and a second rate was recorded. Complex II activity was calculated as the difference between the total rate and the malonate-inhibited rate.

**Complex III**—Complex III (ubiquinol:cytochrome c oxidoreductase) activity was assayed using the short-chain Q analog, Q2 as the electron donor, because it gives a lower nonenzymatic (background) rate than Q1. A fully reduced solution of Q2 was prepared (Q2H2, 80% ethanol, HCl, sodium borohydride). Cyclohexane was then added. The reduced Q2H2 was added from a syringe and was broken by covering the cuvette with parafilm. Temperature reequilibration, Q1 was added to initiate the reaction. Reduction of Q1 was followed at 550 nm. The enzyme was assayed at 340 nm for 3 min. Using a extinction coefficient of 6.22 mM−1 cm−1, the rate with mitochondrial protein was 50% of the rate without mitochondrial protein. The rate was determined by subtracting the rate without mitochondrial protein (nonenzymatic) rate was determined by carrying out the above assay without the addition of mitochondrial protein. Both rates were calculated as first order rate constants, and the true enzymatic rate was determined by subtracting the rate without mitochondrial protein (nonenzymatic background) from the rate with mitochondrial protein.

**Complex IV**—To assay complex IV (cytochrome c oxidase) activity, a solution of reduced cytochrome c was prepared. An excess of ascorbic acid was added to the solution and allowed to fully reduce. A Sephadex G50 column was equilibrated with assay buffer, and the reduced cytochrome c solution was pipetted onto the column. Fractions of eluted cytochrome c were collected. Prior to assay, the reduced cytochrome c solution was checked to make sure that it has remained reduced by measuring the absorbance at 550 nm, adding dithionite, and rechecking the absorbance. Mitochondrial protein was added to the reduced cytochrome c solution and the rate recorded. A fully oxidized absorbance value was obtained by adding ferricyanide to the cuvette. Activity was calculated as a first-order constant.

**Western Blot Analysis**

Ilet protein was subjected to reducing SDS-PAGE using 12% Tris-glycine gels. Proteins were electroblotted from the gels onto polyvinylidene difluoride membranes and probed with either an MnSOD antibody (StressGen, Victoria, Canada) or trans-4-hydroxy-2-nonenal (HNE) antibody (generously donated by Dr. Koji Uchida, Nagoya University, Japan), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Enzyme-catalyzed chemiluminescence (ECL) detection was performed by adding substrate ECL-Oxidase and horseradish peroxidase was developed with the ECL kit from Amersham Biosciences and detected with Super RX x-ray films from Fuji Medical Systems (Stamford, CT).

**mtDNA Analysis**

Single-strand Conformation of Polymorphism (SSCP) Analysis—Total DNA was prepared from frozen islets of 15-week IUGR and control animals. To screen for mtDNA mutations, PCR restriction fragment SSCP was carried out. This method is based on the differential electrophoretic mobility of single-stranded DNA under nondenaturing conditions (45, 46). For this study, the region encoding ATPase 6 was chosen.
for SSCP analysis, since there is less sequence divergence in this region (47). DNA from islets of five IUGRs and five controls at 15 weeks of age was PCR-amplified using primers to ATPase 6. Amplifications were performed on 100 ng of template DNA. PCR conditions were as follows: cycle 1, 5 min at 92 °C, 1 min at 55 °C (or the calculated Tm, 5 °C for the respective oligomer pair), 2 min at 72 °C; cycles 2–30, 1 min at 92 °C, 1 min at the calculated annealing temperature, 2 min at 72 °C. PCR products were digested by SfaCl or Accl at 37 °C for 60 min. Electrophoresis was carried out using 6% polyacrylamide gels containing 8 M urea. 3.5-μl aliquots from 25-μl PCRs were mixed with 5.5 μl of formamide and then denatured for 5 min (95 °C) and placed on ice and located into the preformed slots. The electrophoretic separation was carried out on a horizontal electrophoresis apparatus in room temperature. Autoradiography was carried out with an intensifying screen using Kodak X-Omat film for 17 h.

**Sequencing**—To determine whether IUGR induced specific mutations or deletions, sequence analysis was carried out. Total DNA was prepared from islets of IUGR and control fetuses, 1-week, 7-week, and 15-week-old animals, and their respective mothers. PCR was used to amplify the region of the mtDNA genome encoding ATPase subunit 6. Three sets of primers were used (set 1, 7681–8027; set 2, 7965–8347; set 3, 8278–8641). To control for PCR artifacts, amplification was repetitively done on each DNA sample, and the sequences from at least two separate amplifications that matched were used for analysis. Sequencing reactions (20 μl) contained 0.1–0.5 μg of template, 100 pmol of primer, and either 4 or 8 μl of the Big-Dye Terminator Ready Reaction Mix (ABI). Reaction conditions were 96 °C for 1 min and then 25 cycles of 96 °C for 15 s, 50 °C for 10 s, and 60 °C for 4 min. Reaction products were ethanol-precipitated according to instructions supplied by ABI and then analyzed on an ABI DNA sequencer. Sequences were compared with the sequences of each animal’s corresponding mother’s mtDNA sequence. Sequences were also compared with sequences in the mitochondrial DNA sequence data base.

**mtDNA Content**—To determine the amount of nuclear DNA relative to mtDNA, we used quantitative real time PCR. The TaqMan 7900HT sequence detection system was used to perform real time PCR amplification for glyceraldehyde-3-phosphate dehydrogenase and the mtDNA region, ATPase 6. TaqMan probes were labeled with 5’ 6-carboxyfluorescein (fluorescent reporter) and 3’ 6-carboxytetramethylrhodamine (fluorescence quencher). PCR amplifications were carried out in buffer containing 18.6 mM ammonium sulfate, 67 mM Tris base, 2.5 mM MgCl2, 10 mM 2-mercaptoethanol, 0.1% Me2SO, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 600 nM each of forward and reverse primers, 200 nM TaqMan probe, 0.6 units of Platinum Taq polymerase, and 2% Rox reference dye. The real time PCR reactions were performed in triplicate. mtDNA/nuclear DNA ratios were calculated by dividing the mtDNA signal for ATPase 6 by the glyceraldehyde-3-phosphate dehydrogenase signal and expressing the ratio as a percentage of controls set at 100%.

**Gene Expression of Mitochondrial Encoded Genes: NADH-Ubiquinone Oxireductase Subunit 4L, ATP Synthase (Subunit 6), and Cyclooxygenase I**

Total RNA was extracted from islets using RNAzol B (Tel-Test, Inc.). Real time PCR, using glyceraldehyde-3-phosphate dehydrogenase as a standard, was used to determine levels of mRNA.

**Statistical Analysis**

Statistical analyses were performed using analysis of variance and Student’s unpaired t test. Each independent measurement for fetuses was derived from pooled islets of three litters from IUGR and two litters of control rats. Each independent measurement for 1-week-old pups were derived from pooled islets of 1–2 litters from IUGR and 1 litter of control rats. All data were normalized to the ratio of non-β-cell mass to β-cell. These studies were approved by the Animal Care Committee of the Children’s Hospital of Philadelphia and the University of Pennsylvania.

**RESULTS**

As previously reported (16), birth weights of IUGR animals were significantly less than controls (5.33 ± 0.24 versus 7.19 ± 0.29 g, SGA (n = 85) versus control (n = 101), respectively; p < 0.05). By 7 weeks of age, IUGR and control rats had similar weights (Table I). At 1 week of age, glucose and insulin levels were similar in the two groups. Glucose levels remained normal at 7 weeks in IUGR animals; however, fasting insulin levels were modestly elevated compared with controls (Table I). At 15 weeks of age, fasting glucose levels were moderately increased, and insulin levels remained elevated in IUGRs compared with controls (Table I). β-Cell mass in IUGR rats was mildly decreased compared with controls at 7 weeks of age (7.6 ± 1.8 versus 9.5 ± 1.7 mg, IUGR versus control respectively, p < 0.05). By 15 weeks of age, β-cell mass was markedly reduced in IUGR animals (6.0 ± 2.8 versus 12.7 ± 1.6 mg of IUGR versus control, respectively, p < 0.05). The proportion of non-β-cells in the islet increased with age in IUGR animals and was about 50% of total islet cells at 15 weeks (compared with 36% for controls, p < 0.05).

**Insulin Release**—In previous in vivo studies (16), we determined that insulin secretion in response to glucose was impaired in IUGR rats in the newborn period and progressively worsened with age. To determine whether glucose-stimulated insulin secretion remained impaired after removal from a potentially adverse metabolic milieu, insulin secretion was measured in cultured islets (72 h) using static islet incubation. Basal insulin release at 3.3 mM glucose was similar in islets of IUGR and control rats at 1, 7, and 15 weeks of age. However, insulin secretion in response to 16.7 mM glucose was significantly blunted in IUGR animals and progressively worsened with age (Table II). Leucine (20 mM)-stimulated insulin secretion was also markedly impaired in IUGR islets and was nearly 50% of control values at 1 week of age, 40% of controls at 7 weeks of age, and 27% at 15 weeks (p < 0.05) (Table II).

**Mitochondrial Function—**ATP production in response to 16.7 mM glucose stimulation was markedly blunted in fetal IUGR islets (Fig. 1). ATP production was even further impaired in IUGR animals at older ages (Fig. 1). ATP production in the presence of leucine (20 mM) was higher than that observed with glucose in control fetal islets (4.2 ± 0.2 versus 2.1 ± 0.1 pmol/g protein, p < 0.05). However, leucine failed to stimulate ATP production in IUGR fetal islets (0.62 ± 0.01 versus 0.55 ± 0.02, base line versus leucine stimulation, respectively). Leucine also failed to significantly increase ATP production in islets from older IUGR animals.

The mitochondrial electron transport chain consists of the partially mitochondrial encoded complexes I, III, and IV and the exclusively nuclear encoded complex II. The enzymatic
activities of complex I and III (normalized to citrate synthase activity/β-cell mass) were significantly lower in IUGR compared with control islets and declined with age (Fig. 2). This decrease was also observed in the combined activity of complexes I + III (p < 0.05 versus control, data not shown). In contrast, there was no decrease in the enzyme activity of the nuclear encoded complex II or in the partially mitochondrial encoded enzyme complex IV. Citrate synthase activity did not differ between IUGRs and controls in fetal, 1-week, and 7-week animals. However, by 15 weeks of age, citrate synthase activity in islets from IUGR animals was nearly 50% of controls (p < 0.05).

**ROS Production**—The generation of ROS was measured under basal conditions and after the addition of 16.7 mmol/liter glucose to islets. High concentrations of ROS were detected in IUGR fetal islets in basal conditions; the addition of glucose did not further increase ROS production (Fig. 3). After birth, basal levels of ROS in IUGR islets dropped, but they were still significantly higher than controls. In the presence of 16.7 mM glucose, ROS production was significantly higher than controls at all ages. By 15 weeks of age, ROS production was more than 2-fold higher in IUGR islets compared with controls (Fig. 3).

**Oxidative Stress**—Multiple studies have now shown that intrauterine growth retardation is associated with increased oxidative stress in the human fetus (49–54). Both acute and chronic production of ROS are associated with large increases in the level of MnSOD protein, suggesting that mitochondria have mounted antioxidant defenses (55). Western blot analysis of islet protein showed that the amount of MnSOD protein progressively increased in IUGR islets compared with control animals (Fig. 4). Oxidative stress can also lead to the formation of the highly reactive aldehyde HNE, which is produced when ω6-polysaturated fatty acids undergo free radical-mediated peroxidation (56–58). HNE can react with sulfhydryl groups and histidine and lysine residues of proteins to form covalently modified proteins that can be detected in immunoblots using an antibody directed against the HNE moiety. Detection of HNE-protein adducts is therefore accepted as presumptive evidence of oxidative stress. Western blot analysis revealed multiple bands, suggesting that a number of proteins were modified by HNE in IUGR animals (Fig. 4).

**Mitochondrial DNA**—SSCP was used as a screening method to determine whether mtDNA mutations were present in islets of IUGR rats at 15 weeks of age. The region of the mitochondrial genome encoding ATPase subunit 6 was analyzed, because this particular region has minimal sequence divergence, even between different rat strains (47). Islet DNA from five IUGR and control rats was PCR-amplified. As can be seen in Fig. 5, SSCP analysis of IUGR rat DNA showed shifted and additional bands, suggesting that mtDNA from IUGR rats contains point mutations.

To further characterize mtDNA mutations in IUGR animals, the region of the mitochondrial genome encoding ATPase 6 was sequenced after PCR amplification. Sequence analysis of mtDNA from islets of mothers of IUGR animals and of the mothers of control animals was also performed and used as the referent sequences for their respective offspring. There was no sequence divergence between maternal rats and their IUGR offspring at younger ages (fetal and 1 week). However, we

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**Insulin secretion in islets from control and IUGR animals is shown. Data are the mean ± S.E. of five experiments. Results (mean ± S.E.) are expressed as microunits of insulin released per μg of β-cell mass.**

<table>
<thead>
<tr>
<th>Age</th>
<th>Control glucose</th>
<th>IUGR glucose</th>
<th>Control leucine (20 mM)</th>
<th>IUGR leucine (20 mM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3.3 mM</td>
<td>16.7 mM</td>
<td>3.3 mM</td>
<td>16.7 mM</td>
</tr>
<tr>
<td>1 week</td>
<td>1.05 ± 0.01</td>
<td>3.15 ± 0.40</td>
<td>0.86 ± 0.13</td>
<td>1.54 ± 0.15*</td>
</tr>
<tr>
<td>7 weeks</td>
<td>2.79 ± 0.14</td>
<td>7.65 ± 0.67</td>
<td>2.35 ± 0.21</td>
<td>3.64 ± 0.34*</td>
</tr>
<tr>
<td>15 weeks</td>
<td>1.82 ± 0.09</td>
<td>6.47 ± 0.60</td>
<td>1.13 ± 0.11</td>
<td>1.63 ± 0.12*</td>
</tr>
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* p < 0.05 versus control. n = 22 each group at 1 week; n = 15 each group at 7 weeks and 15 weeks.

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**Mitochondrial Function in IUGR Islets**

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**Table II**

**Glucose- and leucine-induced insulin release**

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**Fig. 1. ATP production in cultured islets.** Values are means ± S.E. of five measurements for each age group. Hatched bars, control; solid bars, IUGR. *, significant difference at p < 0.05 versus control.

**Fig. 2. Enzymatic activity of electron transport chain complexes.** Activities for complex I and complex III were normalized to citrate synthase activity and presented as percentages of control values (n = 3 measurements/age/group). Values are normalized for the ratio of β-cell to non-β-cell mass. *, significant difference at p < 0.05 versus control.

**Fig. 3. ROS production in the presence of 16.7 mM glucose in cultured islets of IUGR rats.** Data are expressed as percentage of control, and values are ± S.E. (n = 3 measurements/age/group). Values are normalized for the ratio of β-cell to non-β-cell mass. *, significant difference at p < 0.05 versus control.
detected numerous mtDNA point mutations (average 3.52 ± 0.31 /H11006 0.31 ± 0.31 /H11003 10 /H11002 3-bp mutations in 7-week-old IUGR rats and 15.21 ± 1.22 /H11006 1.22 ± 1.22 /H11003 10 /H11002 3-bp mutations in 15-week-old rats), some of which were predicted to disrupt ATPase activity based on altered amino acid sequence. Mutations included transition, transversion, and single base insertions and were randomly distributed along the ATPase 6 gene. Thus, there were no obvious mutational hot spots in this particular mitochondrial encoded gene.

We hypothesized that damage to mtDNA would result in a reduction of the available template for PCR, resulting in a reduction in total mtDNA. The mtDNA/nuclear DNA ratio of the controls was set at 100%, and mtDNA damage was expressed as a percentage of this control value. Thus, a lower mtDNA/nuclear DNA ratio represents less initial template, denoting a decrease in the integrity of mtDNA. Mitochondrial DNA-nuclear DNA ratios were significantly higher in IUGR fetuses compared with controls, probably reflecting a compensatory response to the in utero insult. However, after birth, there was a progressive reduction in mitochondrial DNA levels in IUGR islets, and by 15 weeks of age, mtDNA content was reduced by 19% in IUGRs versus controls (Fig. 6).

To determine whether a reduction in mtDNA would be associated with a reduction in mRNA levels of mitochondrial encoded genes, we performed real time PCR analysis. mRNA levels of ATPase 6 were modestly lower in IUGR islets at 1 week of age (60% of control values, p < 0.05 versus IUGR versus control). There was a steady decline in ATPase 6 transcript levels as IUGR animals aged, and at 15 weeks, mRNA levels for this gene were approximately one-third of control levels. mRNA levels of two other mitochondrial encoded genes, NADH dehydrogenase subunit 4L and cyclooxygenase I, were also significantly reduced at 15 weeks in IUGR islets compared with controls (Fig. 7). Thus, decreased mitochondrial gene transcription in IUGR animals is generalized to other mitochondria-encoded genes.

DISCUSSION

These studies are the first to elucidate a molecular mechanism responsible for progressive beta cell dysfunction in IUGR animals. The results show clearly that an altered metabolic intrauterine milieu can induce permanent mitochondrial dysfunction. A vicious cycle results when impaired mitochondria produce elevated levels of ROS that further damage mtDNA, resulting in even higher levels of ROS and more mtDNA deletions. Once the animals develop hyperglycemia, a self-reinforcing cycle of progressive deterioration in mitochondrial function leads to a corresponding decline in β-cell function. The onset of diabetes ensues when a critical level of abnormal β-cell insulin...
secretion combined with β-cell loss is reached. Uteroplacental insufficiency, caused by such disorders as preeclampsia, maternal smoking, and abnormalities of uteroplacental development, is the most common cause of fetal growth retardation in the western world. The resultant abnormal intrauterine milieu restricts the supply of crucial nutrients to the fetus, thereby limiting fetal growth. A major consequence of limited nutrient availability is an alteration in the redox state in susceptible fetal tissues leading to oxidative stress (49–54). In particular, low levels of oxygen, evident in growth-retarded fetuses, will decrease the activity of complexes of the electron transport chain, which will generate increased levels of ROS (59–64).

Overproduction of ROS initiates many oxidative reactions that lead to oxidative damage not only in the mitochondria but also in cellular proteins, lipids, and nucleic acids. Increased ROS levels activate the iron-sulfur centers of the electron transport chain complexes and tricarboxylic acid cycle aconitase, resulting in shutdown of mitochondrial energy production (65). This is the likely etiology for the preferentially lowered Complex I and III activity in IUGR islets, since these complexes contain many subunits with iron-sulfur centers in contrast to the other complexes, which contain far fewer. Furthermore, a damaged mitochondrion generates more ROS than an intact organelle (66–70). As the IUGR animal ages, sustained hyperglycemia will exacerbate mitochondrial dysfunction.

Another manifestation of the progression in oxidative stress in IUGR animals was the accumulation of mtDNA mutations in islets. mtDNA is more susceptible to damaging agents such as ROS than is nuclear DNA (71) because of the low efficiency of mitochondrial DNA repair systems, lack of protection by histones, and unique structural characteristics that may favor mutational events (72, 73). Although not precisely quantifiable, mutations could easily be detected by direct DNA sequencing of the product suggesting that a high proportion of mtDNA is mutated

A substantial reduction in mtDNA copy number was present in IUGR islets, which was associated with a progressive decrease in expression of the mitochondrial encoded genes, ATPase 6, NADH dehydrogenase subunit 4L, and cytochrome oxidase I in IUGR islets. Mitochondrial DNA depletion is also likely to be due to oxidative injury as has been demonstrated in muscle and liver of aged animals (74). The reduction in mtDNA template may in part be the reason that mRNA levels of ATPase 6 were reduced in IUGR islets of older animals. However, in the IUGR newborn, we observed decreased ATPase 6 expression despite a normal mtDNA copy number, indicating that oxidative stress has independent effects upon mitochondrial gene expression in IUGR rats. Similar discrepancies between mitochondrial DNA copy number and transcript levels have been found in muscle of aged animals (74).

It is not clear whether mtDNA mutations contribute to impaired complex I activity and diminished ATP production in IUGR islets. There has been a great deal of controversy regarding the threshold of mtDNA mutations and mitochondrial DNA copy number necessary to compromise the oxidative capacity of the cell. A number of studies suggest that a significant depletion of mitochondria must occur before oxidative phosphorylation is significantly impaired (75). However, more recent studies using magnetic resonance spectroscopy demonstrate that there can be biochemical defects even at very low levels of mtDNA mutations (76, 77). Supporting this hypothesis is the finding that individuals with the A3243G mitochondrial mutation have a very low percentage of mutated to wild-type mtDNA (heteroplasmy), averaging only 20–30% in β-cells (78), yet these patients have a profound β-cell defect. Therefore, it is possible that the combination of ongoing oxidative stress and mitochondrial DNA damage is responsible for diminished mitochondrial respiratory activity in IUGR islets.

There are now substantial data that show that oxidative stress plays a significant role in the progression of β-cell deterioration in type 2 diabetes (28, 31–42, 79–81). In vivo and in vitro studies have linked oxidative stress to loss of glucose-stimulated insulin secretion (31, 34, 36, 82, 83), impaired β-cell differentiation (38), and decreased expression of key genes regulating β-cell function and proliferation (33, 36–39, 84). Whereas oxidative stress was induced either by high levels of glucose or hydrogen peroxide, a recent report shows that even subtle elevations in glucose in vivo can increase the production of ROS by mitochondria in islets of Zucker diabetic fatty rats, an animal model of type 2 diabetes mellitus (85).

Normal mitochondrial function is especially critical to the pancreas as β-cells secrete insulin in response to the generation of ATP through metabolism of glucose (reviewed by Newgard and McGarry (21)). Mitochondrial metabolism of glucose is obligatory for the initial and the sustained insulin secretory responses to glucose (24, 25). Therefore, the impairment in glucose-stimulated insulin secretion in newborn IUGR animals is probably due to impaired production of ATP by the mitochondria. Our finding that leucine-stimulated insulin secretion was blunted in IUGR animals further supports this hypothesis. Leucine is metabolized to produce ATP primarily through the mitochondrial oxidative phosphorylation pathway. Thus, a decreased response to leucine stimulation is due to the disruption of this pathway and indicates a general impairment of mitochondrial function. Our studies are likely to have important clinical applicability as well. A recent study in young adult offspring of patients with type 2 diabetes demonstrated that those subjects with insulin resistance had a 30% reduction in mitochondrial phosphorylation compared with age-matched controls (86). The investigators speculated that insulin resistance in the skeletal muscle of insulin-resistant offspring of patients with type 2 diabetes was due to an inherited defect in mitochondrial oxidative phosphorylation (86). Thus, our studies elucidate the molecular mechanisms underlying the link between mitochondrial dysfunction, whether hereditary or secondary to an environmental insult, and the later development of type 2 diabetes.

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Progressive Accumulation of Mitochondrial DNA Mutations and Decline in Mitochondrial Function Lead to β-Cell Failure
Rebecca A. Simmons, Irena Suponitsky-Kroyter and Mary A. Selak


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