Oligonucleotide Microarray Analysis Reveals PDX1 as an Essential Regulator of Mitochondrial Metabolism in Rat Islets*

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Mutations in the transcription factor IPF1/PDX1 have been associated with type 2 diabetes. To elucidate β-cell dysfunction, PDX1 was suppressed by transduction of rat islets with an adenoviral construct encoding a dominant negative form of PDX1. After 2 days, there was a marked inhibition of insulin secretion in response to glucose, leucine, and arginine. Increasing cAMP levels with forskolin and isobutylmethylxanthine restored glucose-stimulated insulin secretion, indicating normal capacity for exocytosis. To identify molecular targets implicated in the altered metabolism secretion coupling, DNA microarray analysis was performed on PDX1-deficient and control islets. Of the 2640 detected transcripts, 70 were up-regulated and 56 were down-regulated. Transcripts were subdivided into 12 clusters; the most prevalent were associated with metabolism. Quantitative reverse transcriptase-PCR confirmed increases in succinate dehydrogenase and ATP synthase mRNAs as well as pyruvate carboxylase and the transcript for the malate dehydrogenase gene, a subunit of the NADH dehydrogenase comprising complex I of the mitochondrial respiratory chain. As a consequence, total cellular ATP concentration was drastically decreased by 75%, and glucose failed to augment cytosolic ATP, explaining the blunted glucose-stimulated insulin secretion. Rotenone, an inhibitor of complex I, mimicked this effect. Surprisingly, TFAM, a nuclear-encoded transcription factor important for sustaining expression of mitochondrial genes, was down-regulated in islets expressing DN79PDX1. In conclusion, loss of PDX1 function alters expression of mitochondrially encoded genes through regulation of TFAM leading to impaired insulin secretion.

Type 2 diabetes mellitus is a common severe disease of intermediary metabolism usually caused by both β-cell dysfunction and resistance to the biological actions of insulin on its main target tissues (liver, muscle, and fat). The susceptibility for type 2 diabetes is inherited, but single diabetes genes have only been identified in about 5% of cases. Mutations in one of these genes, the homeodomain transcription factor ipf1 (also known as, pdx1, idx1, or stf1), have been associated with a rare form of maturity onset diabetes of the young (MODY4) as well as predisposing individuals to late onset type 2 diabetes (1–5). Furthermore, homozygous null mutations in the ipf1 gene result in pancreas agenesis indicating that this transcription factor is indispensable for both pancreas development and subsequent β-cell function (6, 7).

As in humans, inactivation of both pdx1 alleles in murine models results in pancreas agenesis, whereas heterozygous mice or animals carrying a β-cell-specific deletion of the gene exhibit impaired glucose tolerance (8–11). Several of these models also develop overt diabetes with age indicating a progressive deterioration of β-cell function and/or mass. A recent study concluded that reduction in PDX1 expression impedes primarily β-cell function such as NAD(P)H generation, mitochondrial metabolism, and/or mobilization of intracellular calcium (12) rather than β-cell mass. In contrast, another publication (13) demonstrates that the metabolism secretion coupling (glucose sensing, NADH formation, and calcium currents) was normal in Pdx1<sup>−/−</sup> β-cells. However, β-cells in aging mice were more susceptible to apoptosis, with reduced expression of anti-apoptotic genes such as Bcl<sub>2</sub> and Bcl-2, as compared with control islets. This study concluded that decreased β-cell mass rather than function is the key mechanism whereby PDX1 deficiency leads to impaired blood glucose regulation and to progressive age-dependent development of diabetes (13). The two investigations highlight the profound regulatory complexity that PDX1 impinges on β-cell function and/or survival. The direct contribution of PDX1 on these two potential nonmutually exclusive physiological processes remains to be elucidated.

Whereas PDX1 is clearly a central player in pancreas development and subsequent β-cell function, relatively few bona fide PDX1 target genes have been identified. Most interesting, although PDX1 was found to be one of the main transcription factors mediating the stimulatory effect of glucose on insulin gene expression, its impact on constitutive expression of the hormone in <i>vivo</i> remains controversial (12, 14). Similarly, conflicting results have been reported for the regulation of glucokinase gene expression by the transcription factor (12, 15, 16). In contrast, PDX1 has been consistently shown to regulate transcription of GLUT2, IAPP, and FGFR1 in β-cells as well as somatostatin in δ-cells (17–20). Identification of additional PDX1-regulated genes in β-cells could reveal novel targets.

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¶ The abbreviations used are: MODY, maturity onset diabetes of the young; PDX1, pancreatic duodenal homeobox 1; OXPHOS, oxidative phosphorylation; IBMX, isobutylmethylxanthine; RT, reverse transcriptase.
directly implicated in either impaired insulin secretion and/or apoptosis associated with the MODY4 phenotype.

DNA microarray technology has become a powerful tool to decipher the complex genetic networks altered in response to environmental insults and disease. Here we use this technology to study the impact of PDX1 on β-cell physiology by comparing global gene profiles of islets infected with a viral vector expressing a dominant negative variant of PDX1 (AdRIPDN79PDX1) to control LacZ-infected islets. We identified 94 genes and 32 ESTs that reached a greater than 1.6-fold increase or decrease in expression levels after infection with AdRIPDN79PDX1. Most interesting, expression of the mitochondrially encoded nad1 gene, a subunit of the respiratory chain complex I, NADH dehydrogenase, was decreased by 2-fold. Possibly through a compensatory mechanism, there was increased expression of succinate dehydrogenase and ATP synthase (complex II and V, respectively) as well as genes involved in the anaplerosis pathway. Decreased total cellular ATP as well as a refractory increase in glucose-induced ATP synthesis accompanied these alterations. Moreover the nuclear-encoded transcription factor TFAM, which maintains mitochondrial gene expression, was down-regulated by DN79PDX1. Our study provides a novel role of PDX1 in mitochondrial metabolism through regulation of mitochondrial genes.

MATERIALS AND METHODS

Rat Islet Isolation and Culture—Seven-week-old male Wistar rats (250 g) were purchased from Elevage-Janvier (Le Genest-St-Isle, France). Pancreatic islets were isolated by collagenase digestion, handpicked, and maintained in 11.1 mM glucose/RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (Brunschiwig AG, Basel, Switzerland), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma) (21).

Recombinant Adenoviral Construction and Islet Transduction—The mouse polyclonal cDNA lacking the transcriptional domain of PDX1 (amino acids 1–79) was kindly provided by Dr. H. Wang (16). The recombinant adenovirus AdRIPDN79PDX1 harboring the rat insulin 1 promoter and coding sequence of DN79PDX1 was constructed as described previously (22). AdLacZ, containing the bacterial β-galactosidase cDNA, was used as a control adenovirus (21). Eighteen hours post-isolation, islets were infected with various amounts of recombinant adenoviruses (AdCaLacZ, 17 × 10^5 viral particles/islet and 4 and 17 × 10^6 viral particles/islet for AdRIPDN79PDX1) for 90 min, washed, and cultured in RPMI medium for an additional 48 h.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay—Nuclear extracts with protein extracts were prepared from AdCaLacZ or AdRIPDN79PDX1-infected islets according to the protocol of Schreiber et al. (23). A double-strand oligonucleotide corresponding to the FARFLET element of the rat insulin 1 promoter was radioactively labeled by filling in the ends using the Klenow fragment of DNA polymerase I in the presence of [α-32P]dCTP and purified using the QiAquick nucleotide removal kit (Qiagen AG, Basel, CH). DNA binding assays were performed as described previously (24).

Immunoblot Analysis—Nuclear protein extracts (10 and 20 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred electrophotically to a polyvinylidene difluoride membrane. The membrane was blocked with 5% Topblock and 0.4% Tween 20 in phosphate-buffered saline for 1 h and then left overnight at 4 °C in the presence of a rabbit polyclonal anti-PDX1 antibody (28, 29). Goat anti-rabbit IgG antisera conjugated to horseradish peroxidase (1:4000) (Amersham Biosciences) was then added for 60 min, and immunoreactive products were visualized by enhanced chemiluminescence (SuperSignal West Fico, Pierce) followed by exposure to X-Omat Kodak films (Eastman Kodak Co., Rochester, NY) or by exposure to X-Omat Kodak films (Eastman Kodak Co., Rochester, NY).

Insulin Secretion Assay and ATP Measurement—Islets were washed in Krebs-Ringer bicarbonate-HEPES buffer (KRBH: 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 2 mM NaHCO3, 10 mM HEPES, 0.1% bovine serum albumin) and incubated at 37 °C for 60 min in the same buffer supplemented with 2.5 mM glucose. Subsequently, insulin secretion from 15 size-matched islets per condition was measured over a period of 30 min in KRBH containing the indicated stimulators. Total insulin content was extracted using acid ethanol. Insulin was detected by radioimmunoassay using rat insulin as standard and an antibody raised against rat insulin (Linco, St. Louis, MO). Secreted insulin was expressed as percentage of total cellular insulin content.

Total cellular ATP was measured after 10 min of incubation in 2.5 mM glucose using a bioluminescence assay kit according to the manufacturer’s recommendations (ISI II, Roche Diagnostics). Alterations in cytosolic ATP levels in response to 16.5 mM glucose or 100 μM IBMX, 1 μM forskolin, 16.5 mM glucose were monitored using the adenoviral construct AdCaLuc encoding the ATP-sensitive bioluminescence probe luciferase (25). Islets were preincubated at 37 °C for 30 min in KRBH before the addition of 2.5 mM glucose and 500 μM beet luciferin (Promega, Madison, WI). Changes in ATP were then recorded using a FLUOstar Optima apparatus (BMG Lab Technologies) as described previously by Merglen et al. (26).

Glucose Oxidation—Carbon dioxide production derived from glucose oxidation was measured using the multwell 14CO2-capture assay developed by Collins et al. (27). Radioactive carbon dioxide trapped in filter paper was measured using a Beckman LS6500 Scintillation counter (Beckman Instruments, Nyon, Switzerland), and results were expressed as nanomoles of glucose/mg protein/h.

Oligonucleotide Microarray Assay—RNA was extracted from either control/AdCaLacZ or AdRIPDN79PDX1-infected islets using the RNeasy mini kit (Qiagen, Basel, Switzerland). Because of difficulties in obtaining sufficient amounts of RNA from single experiments and to decrease eventual biases caused by biological variations, islets from three separate experiments were pooled for total RNA extraction yielding one sample. A total of 13 samples, two control and one for each sample, were thus generated and used to produce biotinylated cRNAs. Five μg of RNA was converted into double-stranded cDNA using a cDNA synthesis kit (Superscript Choice, Invitrogen) with a special oligo(dT)24 primer containing a T7 RNA promoter site added 3′ to the poly(T) tract. Biotinylated cRNAs were generated from cDNAs using the bioarray high yield RNA transcript labeling kit (Enzo Life Sciences) and subsequently purified with the RNeasy kit (Qiagen). Eighteen μg of cRNA probe derived from each sample (three AdCaLac and three AdRIPDN79PDX1 islet samples) was fragmented and hybridized to six independent Affymetrix Rat Genome U34A arrays (Affymetrix, High Wycombe, UK) by using an Affymetrix GeneChip Fluidics Station 400 and standard protocols. Aliquots of fragmented rat arrays were now available (U34B and –C); these only provide ESTs, whereas the U34A array contains probes representing all currently full-length or annotated genes. However, it is important to note that this array may not cover all of the endocrine pancreas transcriptome. Arrays were visualized on a Agilent 2500 GeneArray scanner, and image files were processed using Microarray Analysis Suite version 5.0 (Affymetrix). The software calculates “signal” values and provides “detection” calls, “present,” “marginal,” or “absent,” for each probe set. Detection calls are determined from statistical calculations of the difference in hybridization signals between perfect match oligonucleotides and their corresponding control mismatch sequence (probe set). Only genes/ESTs with a detection call present in both control/AdCaLacZ and AdRIPDN79PDX1-expressing islets were subsequently used for comparison analysis. Genes/ESTs that were selectively present in either control or DN79PDX1-expressing islets were not detected under these experimental conditions. To identify differentially expressed transcripts, pairwise comparison analyses were carried out with Affymetrix MAS 5.0. Each of the experimental samples (number of experiments) was compared with each of the reference samples (number of reference experiments), resulting in (number of experiments × number of reference experiments) pairwise comparisons. This approach, which is based on the Mann-Whitney pairwise comparison test, allows the ranking of results by concordance, as well as the calculation of significance (p value) of each identified change in gene expression (28). Only genes/ESTs with a detection call present in both control/AdCaLacZ and AdRIPDN79PDX1-expressing islets triplets were subsequently used for comparison analysis. Genes/ESTs that were differentially expressed were then organized and visualized into functional clusters using the GeneSpring software (Silicon Genetics, Redwood City, CA).

Real Time RT-PCR—Total RNA was extracted using the Trizol reagent (Invitrogen), and 1 μg was converted into cDNA as described previously (30). Primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA). PCR products were obtained upon request. Real time PCR was performed using an ABI 7000 Sequence Detection System (Applied Biosystems), and PCR products were detected using the SYBR Green Core Reagent kit. Three distinct amplifications were performed in duplicate for each
transcript, and mean values were normalized to the mean value of the reference mRNA cyclophilin.

Statistical Analysis—Results are expressed as mean ± S.E. Where indicated, the statistical significance of the differences between groups was estimated by Student’s t test.

RESULTS

Adenovirus-mediated DN79PDX1 Gene Transfer—Biochemical and molecular studies on isolated islets derived from transgenic mice bearing a deleted Pdx1 gene have been hampered by limited islet recovery (13). Therefore, we transduced rat islets with a recombinant adenoviral construct encoding a dominant negative variant of PDX1 (DN79PDX1). DN79PDX1, which lacks the transactivation domain but preserves an intact DNA-binding domain, was shown to exert its dominant negative function by competing with endogenous PDX1 for binding to the cognate site in the rat insulin 1 gene promoter (16). To ensure functionality of the transactivation domain but preserves an intact DNA-binding domain, we transduced rat islets with a recombinant adenoviral construct encoding a dominant

limited islet recovery (13). Therefore, we transduced rat islets with AdCaLacZ or AdRIPDN79PDX1, and mRNA levels for insulin as well a GLUT2 were quantified by real time RT-PCR. Both PDX1 target gene transcripts were dose-dependently decreased in cells overexpressing DN79PDX1 reaching levels 30% of those found in control cells (Fig. 1A) (16). The capacity of similar amounts (4 and 17 × 10^5 viral particles/islet comprising ~3000 cells) of AdRIPDN79PDX1 to direct DN79PDX1 expression in islet β-cells was then assessed by Western blot analysis using nuclear extracts isolated from transduced islets. Immunoblotting using PDX1 antisera revealed a band of 31 kDa in islets infected with AdRIPDN79PDX1 but not with the control adenovirus AdCaLacZ. This band was identical to that observed in the cell line rsaβ-DN-PDX1–59 expressing the corresponding DN79PDX1 in a plasmid construct (Fig. 1B) (16). Most interesting, endogenous PDX1 was only detected by using 20 μg of nuclear protein extracts (Fig. 1B). To assess the ability of DN79PDX1 to interact with its cognate DNA-binding element, electromobility shift assays were performed using a radiolabeled rat insulin 1 gene promoter FARFlat element in the presence of the same nuclear extracts. A single predominant retarded complex was detected with extracts derived from islets transduced with AdRIPDN79PDX1 but not with AdCaLacZ (Fig. 1C). A similar migrating complex was also detected in rsaβ-DN-PDX1–59 cell nuclear extracts along with two weaker complexes corresponding to endogenous PDX1 and HNF-1α (31). The interaction of DN79PDX1 with the FARFlat element was confirmed by the supershift of the complex in the presence of a PDX1 antibody (Fig. 1C, lane 5). Taken together, these results suggest that DN79PDX1 is expressed and capable of interacting with a cognate DNA-binding site in islets infected with the adenoviral construct AdRIPDN79PDX1.

Secretagogue-evoked Insulin Secretion Is Blunted in DN79PDX1-expressing Rat Islets—Mutations in the PDX1 gene, which are associated with either MODY4 or late onset diabetes, have been linked to impaired insulin secretion in β-cells (10, 12). These findings have been challenged recently in a study (13) demonstrating that β-cell mass rather than β-cell function dictates glycermia in a transgenic animal model of MODY4. To resolve this discrepancy, we investigated the impact of dominant negative suppression of PDX1 on insulin secretory responses to glucose, leucine, and arginine using islets transduced with AdRIPDN79PDX1. These secretagogues were selected for their action at different and specific levels of the signal transduction cascade: glucose at the primary step, leucine at the mitochondria downstream of glycolysis, and arginine at the late depolarization event (32). Islets were maintained in KRBH containing 2.5 mM glucose for 60 min prior to the addition of the indicated stimulators for 30 min (Fig. 2A). 16.5 mM glucose, 20 mM arginine, as well as 20 mM leucine evoked a 5- and 7-fold increase in insulin secretion in noninfected islets and β-galactosidase-expressing islets, respectively (Fig. 2A). Increasing amounts of AdRIPDN79PDX1 resulted in a dose-dependent decrease in both glucose- and leucine-stimulated insulin release (90 and 60% inhibition for glucose and leucine, respectively, at 17 × 10^5 viral particles/islet). Most interesting, arginine-evoked insulin secretion was unaffected in islets transduced with low amounts of virus, whereas higher concentrations completely blunted the response. Total cellular insulin content was not altered in either AdCaLacZ or AdRIPDN79PDX1-infected islets, and insulin mRNA levels were detected with extracts derived from islets transduced with AdRIPDN79PDX1 (Fig. 1B, lane 3) but not with AdCaLacZ-infected cells. **, p < 0.01. B) 10 μg of nuclear proteins extracted from islets that were infected with either AdCaLacZ (17 × 10^5 viral particles/islet) or increasing amounts of AdRIPDN79PDX1 (4 and 17 × 10^5 viral particles/islet, lanes 3 and 4, respectively) were resolved on a 10% SDS-polyacrylamide gel. In addition, a nuclear protein extract from an INS1-derived clone expressing high levels of DN79PDX1 (rasβ-DN-PDX1–59, lane 1) was added for comparison (16). The expected 31-kDa protein was revealed by immunoblotting using an antibody raised against the C terminus of PDX1. To detect endogenous PDX1, Western blotting was performed with 10 and 20 μg of nuclear protein extracts derived from islets. C, the same nuclear protein extracts (6 μg) were used to perform electrophoretic mobility shift assay in the presence of a γ32P-labeled FARFlat oligonucleotide. Lane 1, rsaβ-DN-PDX1–59; lane 2, 17 × 10^5 AdCaLacZ viral particles/islet; lane 3, 4 × 10^5 AdRIPDN79PDX1 viral particles/islet; and lane 4, 17 × 10^5 AdRIPDN79PDX1 viral particles/islet. * denotes supershift in the presence of the PDX1 antibody (lane 5).

FIG. 1. Adenovirus-mediated expression of DN79PDX1 in rat islets. A, approximately 3 × 10^5 INS-1E cells were infected with the indicated amounts of viral particles (vp) and incubated for 48 h prior to RNA extraction. Insulin and GLUT2 mRNA levels were then determined by quantitative RT-PCR and normalized to the housekeeping transcript, and mean values were normalized to the mean value of the reference mRNA cyclophilin. Values derived from three independent experiments (means ± S.E.) are presented as fold repression as compared with control AdCaLacZ-infected cells. **, p < 0.01. B) 10 μg of nuclear proteins extracted from islets that were infected with either AdCaLacZ (17 × 10^5 viral particles/islet) or increasing amounts of AdRIPDN79PDX1 (4 and 17 × 10^5 viral particles/islet, lanes 3 and 4, respectively) were resolved on a 10% SDS-polyacrylamide gel. In addition, a nuclear protein extract from an INS1-derived clone expressing high levels of DN79PDX1 (rasβ-DN-PDX1–59, lane 1) was added for comparison (16). The expected 31-kDa protein was revealed by immunoblotting using an antibody raised against the C terminus of PDX1. To detect endogenous PDX1, Western blotting was performed with 10 and 20 μg of nuclear protein extracts derived from islets. C, the same nuclear protein extracts (6 μg) were used to perform electrophoretic mobility shift assay in the presence of a γ32P-labeled FARFlat oligonucleotide. Lane 1, rsaβ-DN-PDX1–59; lane 2, 17 × 10^5 AdCaLacZ viral particles/islet; lane 3, 4 × 10^5 AdRIPDN79PDX1 viral particles/islet; and lane 4, 17 × 10^5 AdRIPDN79PDX1 viral particles/islet. * denotes supershift in the presence of the PDX1 antibody (lane 5).
Isolated rat islets were infected with either AdCaLacZ or AdRIPDN79PDX1. Consistent with previous reports (33), only 30% of interrogated sequences were expressed in islets. Comparison of expression profiles between AdCaLacZ- and AdRIPDN79PDX1-infected islets revealed up-regulation by more than 1.6-fold of 70 genes or ESTs, whereas 56 were down-regulated by more than 1.8-fold (Table I). A detailed description of genes classified according to function along with their average fold change calculated from the triplicate analysis are also listed in Table II. It is noteworthy that, similar to INS-1 cells, GLUT2 mRNA levels were suppressed in islets expressing DN79PDX1. In contrast, the insulin transcript was unchanged (data not shown). Expression of 24 genes encoding metabolic enzymes was altered corroborating the hypothesis that metabolism–secretion coupling is impaired by PDX1 suppression. We therefore chose to further investigate the regulation and cellular impact of transcripts within this functional cluster.

**PDX1 Regulation of Islet Metabolism** — Components from both the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS) represented the largest subgroups of metabolic genes influenced by expression of DN79PDX1 in islets (12 of 24 genes). Consistent with the microarray analysis, real time RT-PCR confirmed that pyruvate carboxylase, cytosolic and mitochondrial malate dehydrogenase, succinate dehydrogenase (subunit A), fumerase, as well as ATP synthase (subunit 6) were up-regulated by ~1.5–2-fold in islets infected with AdRIPDN79PDX1 as compared with control (Fig. 3A). The tricarboxylic acid transporter, which transports malate across the inner mitochondrial membrane, was also increased by 2-fold. Most interesting, quantification by real time RT-PCR revealed a lower induction level for this mRNA as compared with the result obtained by the microarray analysis (see Table II). Finally, a 60% decrease in mRNA levels of the ND1 subunit of NADH dehydrogenase (complex I of the OXPHOS) was measured in infected islets (Fig. 3A). Fig. 3B summarizes the action of individual proteins within the tricarboxylic acid cycle and OXPHOS. The abnormalities associated with expression of DN79PDX1 suggest that pyruvate is shunted toward malate, which is subsequently transported out of the mitochondria. This cellular adaptation appears to result from the reduced capacity of complex I of the respiratory chain to accept electrons from NADH. Concomitantly, succinate dehydrogenase, which forms complex II and receives electrons from FADH₂, is increased to compensate for the lack of reducing power derived from NADH. Ultimately, less ATP will be synthesized explaining the compensatory up-regulation of ATP synthase (subunit 6) in an attempt to maintain ATP production.

To confirm the physiological impact of these alterations, we measured total cellular ATP levels in AdCaLacZ or AdRIPDN79PDX1-transduced islets in the presence of 2.5 mM glucose. As predicted from gene profiling, basal ATP levels were 4-fold lower in islets expressing DN79PDX1 as compared with control LacZ islets (Fig. 4A). We next investigated whether glucose was still capable of raising cytosolic ATP levels in DN79PDX1-expressing cells using the ATP-sensitive bioluminescence probe luciferase (25). Addition of 16.5 mM glucose to control/LacZ islets resulted in a 23% increase of cytosolic ATP, which was sustained until the addition of azide (Fig. 4B, left side of graph; compare the circle delineated trace to the triangle delineated trace). In contrast, glucose-induced cytosolic

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**Microarray Analysis of DN79PDX1-infected Rat Islets**

**Fig. 2.** Secretagogue-induced insulin secretion but not glucose oxidation is impaired in rat islets overexpressing DN79PDX1. Isolated rat islets were infected with either AdCaLacZ (17 × 10⁵ viral particles/islet) or increasing amounts of AdRIPDN79PDX1 (4 and 17 × 10⁵ viral particles/islet, A and B, respectively) for 90 min and subsequently cultured for 48 h in 11.5 mM glucose/RPMI 1640. A, insulin secretion was assessed in 30-min static incubations in response to 2.5 or 16.5 mM glucose; 20 mM arginine/5 mM glucose, or 20 mM leucine. Insulin released in KRBB was quantified by radioimmunoassay and expressed as a percentage of total cellular insulin content. Results are the mean ± S.E. of eight independent experiments performed in duplicate. *, p < 0.05, B, insulin secretion was also measured in islets exposed to 1 µM forskolin and 100 µM IBMX in the presence of 2.5 or 16.5 mM glucose. C, [U-¹⁴C]Glucose oxidation was measured in islets overexpressing LacZ or DN79PDX1. Islets were preincubated for 30 min in KRBB solution containing 2.5 mM glucose and subsequently cultured in KRBB with [U-¹⁴C]glucose at the indicated concentrations for 2 h. CO₂ released was measured as described under “Materials and Methods.” Data are the mean ± S.E. of four independent experiments performed in triplicate and are expressed as nanomoles of oxidized glucose/mg of protein/h. ***, p < 0.01.

AdRIPDN79PDX1-transduced islets suggesting that reduced insulin secretion was not because of decreased proinsulin biosynthesis (data not shown). Most interesting, the secretory response to high glucose in PDX1-deficient islets was restored in the presence of 1 µM forskolin and 100 µM IBMX (Fig. 2B). This suggests that the exocytotic machinery is fully functional. To further assess metabolism–secretion coupling, we estimated the rate of glucose oxidation by measuring the conversion of [U-¹⁴C] glucose to [¹⁴C]CO₂ in islets expressing DN79PDX1. Formation of [¹⁴C]CO₂ from 15 mM [U-¹⁴C]glucose was equally efficient in both control and transduced islets (4-fold increase, Fig. 2C). Taken together, these results support the hypothesis that reduction in PDX1 impairs mitochondrial fuel-mediated insulin secretion without altering glucose oxidation. Furthermore, membrane depolarization-induced calcium rises may also be inefficient in causing insulin exocytosis, as indicated by the attenuated effect of arginine.
ATP formation was completely blunted in islets expressing DN79PDX1 (Fig. 4B, left side of graph, black square). ATP levels in 16.5 mM glucose did not decrease as quickly as islets maintained in 2.5 mM glucose indicating that ATP was being formed albeit at much lower rates than in control islets. Corroborating this finding, addition of azide, which dissipates the mitochondrial membrane potential and thus ATP formation, resulted in the inhibition of ATP generation in both control and AdRIPDN79PDX1-transduced islets. ATP formation by glucose was not potentiated by the addition of forskolin and IBMX in control islets (Fig. 4B, right side of graph). Surprisingly, in DN79PDX1-expressing islets glucose caused a small increase (8%) in the presence of forskolin and IBMX (Fig. 4B, right side of graph); the latter agents did not affect cytosolic ATP at 2.5 mM glucose (data not shown). These results suggest that the generation of cAMP permits glucose to cause a small rise in cytosolic ATP. Through different processes, cAMP and ATP will restore synergeize to glucose-induced insulin secretion.

We next used rotenone, a known inhibitor of the mitochondrial electron transport chain complex I (34), to determine whether we could recapitulate the effect observed with DN79PDX1 on glucose-induced ATP synthesis. Such inhibitors have been shown previously to lower total islet ATP and suppress glucose and potassium-stimulated insulin secretion (35). Rotenone dose-dependently inhibited cytosolic ATP generation from glucose attaining complete suppression at 5 μM of the pesticide (Fig. 4D). The 5 μM rotenone trace was similar to the one generated by islets infected with AdRIPDN79PDX1, supporting the concept that complex I is a target of the transcription factor.

**TFAM Is Regulated by PDX1**—Our results suggest that the primary β-cell defect associated with reduction in PDX1 expression is altered in ATP synthesis through suppression of the ND1 subunit of complex I. Intriguingly, the nd1 gene is mitochondrially encoded, whereas PDX1 regulates nuclear gene expression. The common link between these two mutually exclusive genes could implicate the mitochondrial transcription factor TFAM (36). This factor is nuclear-encoded and translocates to the mitochondria where it controls mitochondrial gene transcription and could thus be a candidate PDX1 target. Because TFAM is not represented on the U34A array, we performed quantitative real time RT-PCR on RNA isolated from islets infected for 2 and 5 days with either AdCaLacZ or AdRIPDN79PDX1 adenoviruses. TFAM mRNA levels were suppressed by 50 and 60% in DN79PDX1-expressing cells 2 and 5 days postinfection as compared with control islets indicating that the transcription factor is a bona fide PDX1 target (Fig. 5A).

In the long term, reduced respiratory activity caused by repressed mitochondrial gene expression may lead to increased susceptibility of β-cells to apoptosis. To investigate this possibility, the gene expression profile of bclXL, bcl-2, and Cas-3 was evaluated in islets 2 and 5 days after infection. Real time RT PCR revealed a 50% increase in mRNA levels of the proapoptotic gene caspase-3 in islets transduced with AdRIPDN79PDX1 as compared with control islets 48 h after infection (Fig. 5B). However, 5 days postinfection, caspase-3 mRNA levels were not significantly different from those of control islets, whereas the expression of the anti-apoptotic gene bcl-2 was decreased by 40% (Fig. 5B). These results support increased susceptibility of the β-cell to apoptosis.

**DISCUSSION**

The molecular mechanisms connecting reduced PDX1 activity with β-cell dysfunction remain elusive (12, 13). By using a combination of biochemical and molecular tools, we have identified the mitochondria as a primary target of PDX1 regulation. We find that suppression of PDX1 through adenoviral expression of a dominant negative form of the transcription factor alters the transcripts of several mitochondrial proteins important in the coupling of glucose metabolism to insulin secretion. Biochemically, the significance of the mitochondria is highlighted by results demonstrating that forskolin and IBMX can restore the effect of glucose on insulin secretion through production of cAMP. Most interesting, forskolin and IBMX in the presence of glucose did induce a small increase in cytosolic ATP in islets infected with AdRIPDN79PDX1, albeit at very low levels as compared with control islets. This rise may result from an increase in mitochondrial Ca$^{2+}$ derived from intracellular stores causing stimulation of mitochondrial oxidative activity. Such a mechanism was recently demonstrated in Min6 cells exposed to GLP-1, a cAMP-generating hormone (37).

Restoration of glucose-induced insulin secretion by forskolin and IBMX suggests that the exocytotic process is normal in PDX1-deficient islets. Yet our finding that arginine-stimulated insulin secretion is impaired in PDX1-deficient islets would argue against this observation. Indeed, arginine has been shown to stimulate insulin secretion by depolarizing the plasma membrane and to stimulate Ca$^{2+}$ influx through voltage-sensitive Ca$^{2+}$ channels (38). According to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor hypothesis, insulin granule docking, priming, and release in

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**Table I**

Functional clustering of genes that were differentially regulated in DN79PDX1 overexpressing islets as compared with control islets.

| Cluster | No. genes represented on microarray | No. genes regulated |
|---------|-------------------------------------|---------------------|
| Receptors | 1565 | 2 | 3 |
| Transporters | 801 | 1 | 2 |
| Metabolism | 789 | 22 | 2 |
| Signal transduction | 749 | 4 | 1 |
| Nuclear or DNA-binding proteins | 365 | 3 | 13 |
| Growth control and apoptosis | 341 | 5 | 4 |
| Cell adhesion and extracellular matrix | 184 | 3 | 1 |
| Membrane and granule-associated proteins | 86 | 2 | 3 |
| Antioxidants and NO production | 45 | 4 | 1 |
| Chemokines and cytokines | 38 | 1 | 7 |
| Cytoarchitecture | 34 | 1 | 6 |
| Miscellaneous | 39 | 0 | 3 |
| ESTs | 3765 | 22 | 10 |
| Total | 8801 | 70 | 56 |
## Table II

Classified list of known genes identified by Affymetrix microarray analysis as differentially expressed in DN79PDX1-infected islets versus control islets

TCA, indicates tricarboxylic acid cycle; PCNA indicates proliferating cell nuclear antigen.

| Cluster/GenBank™ database | Gene name | fold change |
|---------------------------|-----------|-------------|
| A. Cell adhesion/extracellular matrix | M14656 | Osteopontin (Spp1) | +3.2 |
|                          | M7207 | α1, type I collagen | +3.6 |
|                          | X70369 | α1, type III collagen (Col3a1) | +3.3 |
|                          | D00913 | Intercellular adhesion molecule-1 (ICAM-1) | −2.3 |
| B. Membrane and granule-associated proteins | M27207 | Taurine transporter | −6.4 |
|                          | X76985 | Lactein (carboxypeptidase A inhibitor) | −2.8 |
|                          | S68135 | Glucose transporter GLUT1 | −1.8 |
| C. Receptors | M60103 | Protein tyrosine phosphatase, receptor type, F (Ptpn1) | +1.6 |
|                          | M59814 | Eph receptor B2 | +1.8 |
|                          | L29232 | Insulin-like growth factor 1 receptor (Igflr) | −1.6 |
|                          | AA892417 | Receptor ligand ephrin A1 | −2.1 |
|                          | D14869 | Prostaglandin E receptor 3 (subtype EP3) (Ptger3) | −2.2 |
| D. Transporters | M30581 | ATPase, Ca++-transporting (Atp2a3) (SERCA) | +1.6 |
|                          | M11794 | Metallothionein-2 and -3 protein | +6.9 |
|                          | AI102562 | Metallothionein (Mtla) | +4.4 |
| E. Signal transduction | M40581 | Metallothionein-2 and -3 protein | +6.9 |
|                          | AA892264 | Membrane-associated protein 17 | +6.4 |
|                          | X76985 | Latexin (carboxipeptidase A inhibitor) | +2.8 |
|                          | S68135 | Glucose transporter GLUT1 | +1.8 |
| F. Cytoarchitecture | M23601 | Monoamine oxidase B (Maob) | +1.8 |
|                          | M96601 | Taurine transporter (TaurT) | +2.7 |
|                          | AA892417 | Receptor ligand ephrin A1 | +2.1 |
|                          | D00913 | Intercellular adhesion molecule-1 (ICAM-1) | −2.3 |
| G. Growth control and apoptosis | M24604 | PCNA | +1.7 |
|                          | AJ006971 | Death associated-like kinase (Dapk1) | +1.7 |
|                          | X75207 | Cyclin D1 | +2.0 |
|                          | M60322 | Aldose reductase gene | +1.6 |
|                          | AI102562 | Metallothionein (Mtla) | +4.4 |
| H. Metabolism | U32314 | Pyruvate carboxylase | +2.9 |
| 1. Gluconeogenesis/glucolysis | U32314 | Pyruvate carboxylase | +2.9 |
|                          | M858621 | CaM-kinase II inhibitor α | +2.1 |
|                          | M57664 | Creatine kinase, brain (Ckb) | +2.0 |
|                          | AI102079 | Integrin-linked kinase (Ilk) | +1.7 |
|                          | AF013144 | MAP kinase phosphatase (cpg21) | +1.6 |
| 2. TCA cycle | AI227808 | Microtubule-associated protein 2 (Map2) | +1.9 |
|                          | S76054 | Cytokeratin-8 | +1.7 |
|                          | X81448 | Keratin-18 | +1.9 |
|                          | AI230260 | CaM-kinase II inhibitor (Csnk2b) | +2.1 |
|                          | AI227808 | Microtubule-associated protein 2 (Map2) | +1.9 |
| 3. Oxidative phosphorylation | X59737 | Ubiquitous mitochondrial creatine kinase | +2.5 |
|                          | X54510 | ATP synthase F0 complex, subunit 6 | +2.0 |
|                          | U08976 | NADH-dehydrogenase (Ndi) mitochondrial | −2.2 |
|                          | J05470 | Mitochondrial carnitine palmitoyltransferase II | +2.0 |
|                          | J04473 | Fumarate hydratase (Fh) | +1.6 |
| 4. β-Oxidation | S70011 | Tricarboxylate carrier | +4.8 |
|                          | U3120 | ATP synthase subunit d | +2.0 |
|                          | U171506 | ATP synthase H+ -transporting, mitochondrial F1 complex e subunit | +1.7 |
|                          | D13120 | ATP synthase F0 complex, subunit 6 | +2.0 |
|                          | AA892354 | Isoomurate dehydrogenase 1 (Itdh1) also antioxidant | +1.8 |
|                          | AA892354 | Isoomurate dehydrogenase 1 (Itdh1) also antioxidant | +1.8 |
|                          | AA892354 | Isoomurate dehydrogenase 1 (Itdh1) also antioxidant | +1.8 |
| 5. Carbohydrate | AB006137 | α-1,2-Fucosyltransferase | +3.2 |
|                          | M13962 | Glucuronidase, β (Gusb) | +2.0 |
response to secretagogues involves the formation of a protein core complex between vesicles and the plasma membrane, which requires both Ca\textsuperscript{2+} and ATP-dependent steps (39). No significant changes were detected by gene profiling in transcripts encoding either secretory proteins (39), including members of the synaptotagmin, syntaxin, and Rab3 protein families or components forming voltage-activated calcium channels. These data suggest a fully functional exocytotic machinery.

Mitochondrial OXPHOS consists of five large enzyme complexes (complexes I–V) that couple oxidation to phosphorylation in the inner mitochondrial membrane and provides most of the ATP essential for cell survival. In the pancreatic β-cell, ATP and other mitochondrial factors also accomplish the coupling of glucose metabolism to insulin secretion (32). We found that expression of the nd1 gene, a subunit of the OXPHOS complex I, was reduced by 60% in DN79PDX1-expressing islets as compared with control AdCaLacZ-infected cells. Complex I, also known as NADH dehydrogenase or NADH:ubiquinone oxidoreductase, is composed of 43 different subunits which catalyzes the transfer of electrons from NADH to ubiquinone (41). Seven subunits including ND1 are encoded by the mitochondrial genome and are critical for enzyme activity (42). Mutations in these subunits have been associated with a vari-
ety of neurodegenerative disorders most notably Leber's hereditary optic neuropathy (43). More important, several mutations in the nd1 gene have also been associated with type 2 diabetes (44–47). PDX1-deficient islets exhibited a strong reduction in total cellular ATP content, and the glucose-induced rise in cytosolic ATP was completely abolished, an effect that was duplicated by rotenone. Consistent with these findings, a previous report (35) has demonstrated that Amytal, another inhibitor of complex I, repressed glucose-induced insulin exocytosis. Most interesting, lower mitochondrial respiratory coupling efficiency has been shown to increase reactive oxygen species, which consumes glutathione (48, 49). The rate-limiting enzyme in glutathione biosynthesis, glutamate-cysteine ligase, was up-regulated by 2-fold in the DN79PDX1 islet, corroborating the overall cellular adaptation to abrogated complex I coupling (Table II). Therefore, in the MODY4 phenotype we can postulate that reduced ND1 expression restraints ATP generation and renders cells inept to increased production in response to secretagogues such as leucine and glucose. Our results suggest that in parallel there is a cellular adaptation to increase ATP, reflected by the enhanced succinate dehydrogenase (complex II) and ATP synthase (complex V) mRNA levels. Furthermore, the increased transcript levels for pyruvate carboxylase, mitochondrial malate dehydrogenase, and fumerase may also be a consequence of the reduced complex I activity. These three enzymes are part of the anaplerosis pathway from pyruvate, which is essential for the generation of ATP and other coupling factors in the β-cell (50–52). It can be speculated that this leads to increased conversion of pyruvate to malate which is then transferred to the cytosol via the equally up-regulated tricarboxylic acid carrier. The net result will be reduced tricarboxylic acid cycle activity and concomitantly diminished NADH formation, a phenomenon reported by Brissova et al. (12) in islets isolated from PDX1 heterozygous mice. Despite the postulated increases in the respiratory chain activity beyond complex I and increases in the enzymes of the anaplerosis pathway, cytosolic ATP is not raised by glucose thus providing an explanation for the insulin secretion defect. Most interesting, these alterations occur in the absence of any significant changes in glucose oxidation indicating a compensatory adaptation that maintains overall CO2 production. Consistent with this observation, repressed pyruvate dehydrogenase activity in rat islets exhibited no alterations in CO2 production because of a compensatory increase in pyruvate carboxylase (52).

The observation that a subunit of the mitochondrially encoded enzyme NADH dehydrogenase is inhibited by repression of a nuclear transcription factor led us to postulate that PDX1 may regulate expression of the mitochondrial transcription factor TFAM. This was confirmed by quantitative RT-PCR showing a 50% reduction in TFAM mRNA levels. TFAM interacts with the unique noncoding sequence of the mitochondrial genome, which contains two major transcription initiation sites.

FIG. 3. Overview of alterations in the intermediary metabolism induced by PDX1 dysfunction in rat islets. A, confirmation by quantitative RT-PCR of DN79PDX1-regulated genes involved in mitochondrial metabolism and oxidative phosphorylation. Values were normalized to the housekeeping gene cyclophilin and compared with islets infected with AdCaLacZ (dashed line). Data are the means ± S.E. of four independent experiments performed in triplicate (p < 0.05). The abbreviations used are as follows: PC, pyruvate carboxylase; mMDH, mitochondrial malate dehydrogenase; FU, fumarase; TCC, tricarboxylate carrier; cMDH, cytosolic malate dehydrogenase; NADH, NADH dehydrogenase; Suc DH, succinate dehydrogenase. B, NADH dehydrogenase is repressed, whereas succinate dehydrogenase and ATP synthase are increased to compensate for the deficiency in ATP production. Concurrently pyruvate is shunted toward malate, which exits the mitochondria by the tricarboxylate carrier.
Fig. 4. Cellular ATP levels are reduced in AdRIPDN79PDX1-infected islets. A, total cellular ATP levels were measured in islets overexpressing either LacZ or DN79PDX1 and maintained in 1 mM glucose for 10 min. Results represent the means ± S.E. **, p < 0.01. B, cytosolic ATP production in response to 16.5 mM glucose was determined over a period of 20 min using the ATP-sensitive bioluminescence probe luciferase. Luminescence was recorded in a FLUOstar Optima apparatus. Islets were equilibrated in KBHB buffer for 30 min prior to initiation of recording. Glucose and azide were added at the indicated times (arrows). Results are the mean ± S.E. of at least five experiments performed in duplicate. Cytosolic ATP generation was also measured in response to 16.5 mM glucose in the presence of 1 μM forskolin/100 μM IBMX (n = 5) (C) or increasing concentrations of rotenone (n = 3) (D).

as well as regulatory sequences and sustains the basal transcription of mitochondrial genes (36, 53). The central function of TFAM suggests that other genes would also be repressed in the mitochondria. Indeed, comparison of gene profiles with less stringent criteria (see “Materials and Methods”) revealed that subunits I-III of cytochrome oxidase as well as transfer RNA encoding genes were also down-regulated by -50% in AdRIPDN79PDX1-infected islets. It is noteworthy that β-cell-specific inactivation of TFAM impaired glucose-induced insulin secretion later followed by β-cell loss (54). Because limited sequences for either the human or rat TFAM promoters are currently available, we cannot determine whether PDX1 interacts with the 5′-flanking region of the gene.

Our study clearly indicates that the primary defect associated with impaired glucose-induced insulin secretion in β-cells with reduced PDX1 expression is linked to mitochondrial dysfunction in agreement with previous work on PDX1-deficient mice (12). Persistent reduction in ATP production will compromise not only insulin secretion but also cell viability as demonstrated in the TFAM null mice (54). This notion is substantiated by decreased expression of the anti-apoptotic gene Bcl-2 in islets infected with AdRIPDN79PDX1. Decreased expression of housekeeping genes such as β- and γ-actin as well as the microtubule-associated protein tau is further indicative of apoptosis (Table II). Therefore, subtle changes in energy production, not affecting the stimulation of insulin secretion, may with time promote β-cell apoptosis, as reported for another strain of PDX1 heterozygous mice (13).

In conclusion, the present findings demonstrate that suppression of PDX1 activity has severe consequences on β-cell mitochondria. The in vitro data suggest that impaired mitochondrial function in MODY4 as well as late onset type 2 diabetes may lead to both defective insulin secretion and time-dependent decrease in β-cell mass.

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Fig. 5. The transcription factor TFAM and the anti-apoptotic gene Bcl-2 are decreased in islets expressing DN79PDX1. Total RNA was extracted from islets 2 and 5 days after infection with either AdCaLacZ or AdRIPDN79PDX1. cDNA was synthesized, and mRNA levels for TFAM (A) as well as Bcl-XL, Bcl-2, and caspase-3 (B) were determined by real time PCR using an ABI 7000 sequence detection system. Values were normalized to the housekeeping gene cyclophilin, and fold variations of individual genes were determined by comparison to control samples. Results are the means ± S.E. of three independent experiments performed in triplicate *, p < 0.05.
